EXPRESSION AND CHARACTERIZATION OF TOLL-LIKE RECEPTOR 10

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By

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ABSTRACT

Toll-like receptors (TLRs), named after toll proteins identified in *Drosophila melanogaster*, are the pattern recognition receptors in the innate immune system that detect microbes. TLRs are mono, membrane-spanning, as well as non-catalytic receptors, which are mainly expressed in sentinel cells, such as the dendritic cells, neutrophils and macrophages. While humans have ten TLRs (TLR 1 to 10), the mouse has another three (TLRs 11, 12, 13). TLRs are made up of glycoproteins, which have luminal ligand-binding sites consisting of leucine-rich repeat (LRR) for detection of pathogens leading to activation of immune cells. TLR1, 2, 4, and 6 are responsible for recognition of lipids (such as triacetylated lipopeptide), peptidoglycan, and lipopolysaccharide (LPS). However, the TLR3, 7, 8, and 9 mainly recognize nucleic acids, such as double-stranded RNA (dsRNA) and CpG DNA, while the TLR13 detects ribosomal RNA sequences. So far, there are no data on the localization and immunological functions of TLR10.

I studied the expression, localization and role of TLR10 in *S. pneumoniae* infection. First, I examined the expression of TLR10 in lungs of pig, cattle, dog, rat, and chickens. The light and electron microscopic data show TLR10 expression in vascular endothelium and smooth muscles in lungs of control and inflamed animals. Further, we found altered basal level of expression and localization of TLR10 in bovine neutrophils treated with *E. coli* lipopolysaccharide. These data show the expression of TLR10 in the lungs of tested animal species, and its alteration by LPS in bovine neutrophils.

The next study was designed to investigate the regulation of TLR10 expression and to address its role in neutrophil chemotaxis. *E. coli* LPS activated human neutrophils showed temporal and spatial change in TLR10 expression. Confocal microscopy showed

cytosolic and nuclear distribution of TLR10 in normal and activated neutrophils. TLR10 in *E. coli* LPS-activated neutrophils colocalized with flotallin-1, a lipid raft marker, and EEA-1, an early endosomal marker, suggested its endocytosis. Live cell imaging of LPS activated neutrophils showed TLR10 translocation to the leading edge. Neutrophils upon TLR10 knockdown were unable for fMLP-induced migration. TLR10 knockdown reduced the number of membrane pseudopods in activated neutrophils without altering the expression of key proteins of actin nucleation process, ARP-3 and Diap1. These data show TLR4-mediated pathway for regulation of TLR10 expression, and that TLR10 may have a role in neutrophil chemotaxis.

Next, I examined the role of TLR10 in innate immune response to *S. pneumoniae* infection in U937 human macrophage cell line. *S. pneumoniae* are major causative agents of pneumonia, meningitis and bacteremia. A significant increase in TLR10 mRNA expression was found in *S. pneumoniae* (10^7 cfu for 6hr) challenged macrophages. TLR10 knockdown significantly reduced production of IL-1β, IL-8, IL-17 and TNF-α and no significant change in IL-10 expression, and also significantly diminished nuclear translocation of NF-κB but without affecting the phagocytosis of *S. pneumoniae*.

Altogether, I report the that TLR10 is expressed in the normal and inflamed lungs in cattle, pigs, dogs, rats, chickens and humans. The expression of TLR10 is altered in activated neutrophils, and it plays a role in neutrophils chemotaxis and production of pro-inflammatory cytokines in macrophages infected with *S. pneumoniae*.

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ALI	Acute lung injury
AM	Alveolar macrophages
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ADCC	Antibody-dependent cellular cytotoxicity
BALF	Broncho-alveolar lavage fluid
BSA	Bovine serum albumin
CLI	Chronic lung injury
CLR	C type lectin receptors
CRP	C-reactive protein
DAMP	Damage associated molecular patterns
DISC	Death-inducing signaling complex
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
FADD	FAS-associated death domain
GRK2	G protein coupled receptor kinase 2
ICAM	Intercellular adhesion molecule
IHC	Immunohistochemistry
IRAK	IL-1R-associated kinases
LBP	LPS Binding Protein
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide

LSP-1	Leukocyte specific protein 1
MCP-1	Monocyte chemotactic protein-1
MyD88	Myeloid differentiation primary-response protein 88
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
NLR	NOD-like receptors
PAMP	Pathogen associated molecular pattern
PIM	Pulmonary intravascular macrophage
PRR	Pathogen recognition receptor
PSGL-1	P-selectin glycoprotein ligand 1
RAS	Renin-angiotensin-system
RIG	Retinoid acid inducible gene-1
RLR	RIG-1- like receptors
ROS	Reactive oxygen species
SDF-1	Stromal derived Factor 1
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TAB1	TAK1-binding protein 1
TAK1	Transforming growth factor β activated kinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TGF-β	Transforming growth factor beta
TRADD	TNFR-associated death domain

TRAF6	Tumor-necrosis factor receptor-associated factor 6
TRAIL	TNF-related apoptosis inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon
UFCB	ultrafine carbon particles
VCAM	Vascular cell adhesion protein-1
vWF	von Willebrand factor

CHAPTER. 1 REVIEW OF LITERATURE

1.1 Introduction

The respiratory tract continuously samples the outer environment and is a primary site for the pathogen entry into the body. It has a surface area of approximately $75m^2$ in humans and failure to clear pathogens from this area will lead to airway inflammation or respiratory tract infection (Smith, 1994). Before the discovery of DNA sequencing and ribotying, the trachea and pharyngeal area were thought to be a sterile environment in a normal organism because of ineffective methods to culture and isolate microorganisms from these sites. Lung inflammation caused by lung microbiota disproportion or invading pathogens results in significant morbidity and mortality in human and animal health care (Lambrecht and Hammad, 2015). According to the annual reports of the Asthma Society of Canada, the economic burden of respiratory illnesses is $5.9 billion annually (Asthma.ca, 2011).

In humans, respiratory diseases and lung inflammation lead to clinical conditions such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and chronic lung injury (CLI) causing significant morbidity and mortality. ALI is characterized by disruption of endothelial and epithelial barriers, infiltration of immune cells, such as neutrophils, to the site of injury, and a life-threatening escalation of lung inflammation (Donahoe, 2011). Chronic inflammatory lung diseases such as asthma are characterized by airway hyper-responsiveness leading to airway tissue remodeling and reduced lung function (Johnson and Matthay, 2010).

The first line of defense against the microbial invasion of the respiratory system is composed of mechanical barriers such as the mucociliary layer composed of ciliated cells, and secretions of goblet cells and sub-epithelial glands (Nicod, 2005). The innate immune system has a variety of cells expressing pathogen recognition receptors (PRRs) to recognize the pathogen associated molecular patterns (PAMPs) of pathogens that escape mechanical barriers (Iwasaki and Medzhitov, 2015). PRRs, such as toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-1- like receptors (RLRs) are designed to recognize pathogens and trigger the host immune response (Iwasaki and Medzhitov, 2015).

Toll receptors were first identified in Drosophila as a receptor associated with embryonic pattern development (Anderson et al., 1985; Hashimoto et al., 1988). A decade later these receptors were reported to have immune reactivity against fungal infection (Medzhitov et al., 1997). Mammalian homologue of Toll proteins, defined as Toll-like receptors, were identified and reported to play a role in bacterial LPS-mediated inflammation (Takeuchi and Akira, 2002). Currently, 13 TLRs have been identified with each TLR executing innate immune responses. TLRs also recognize intracellular ligands including proteins and peptides, polysaccharides, peptidoglycans, nucleic acids, and phospholipids, which are critical structural building blocks of cells and their extracellular matrix (Beutler, 2009).

The important role of TLRs in the detection of pathogens and progression of inflammation makes them ideal drug target candidates. This body of work is the result of investigating TLR10, a recent addition of TLR family of proteins, whose ligand is still unknown. Specifically, I examined the expression of TLR10 in multiple species,

regulation of its expression and function in neutrophils, and the role of TLR10 in *Streptococcus pneumoniae* infection.

1.2 Respiratory diseases in humans

New or established human respiratory diseases cause significant mortality and morbidity due to drug resistant pathogens and lack of complete understanding of disease pathogenesis. The two clinical conditions affecting the lung that were relevant to this thesis are described below.

1.2.1 Acute lung injury

ALI is a complex respiratory disorder characterized by non-hydrostatic pulmonary edema and acute capillary injury. ALI patients manifest rapid breathing (tachypnea), hypoxemia, loss of lung compliance and pulmonary infiltration of immune cells (Meng et al., 2010). ALI can be caused by an infectious agent, such as bacterial or viral infections, or occur as a sequel to non-pulmonary disease processes, such as acute pancreatitis or sepsis. Since ALI has an acute-onset of noncardiogenic or nonhydrostatic pulmonary edema, defined by a PaO2/FIO2 ratio lower than 300 and acute-onset neutrophil-dominant inflammatory lung disease, the neutrophil-epithelial interactions are important in maintaining the barrier function (Fujishima, 2011). During endotoxin-induced ALI, neutrophils are recruited from the circulation and bone marrow to the lung through the functions of many proteins such as Stromal Derived Factor 1 (SDF1), a chemokine produced by injured epithelium. Specific cytokines (e.g. TNF-α, IFN-γ and IL-8) produced in response to pathogenic invasion regulate the barrier properties of lung microvasculature (Sedwick, 2012; Mouratis et al., 2015). Alveolar epithelial injury

causes reduced surfactant production, which further reduces fluid transport and increases the accumulation of alveolar edema (Matthay and Kronish, 1990). During ALI, the endothelial integrity is damaged and endothelium-dependent relaxation of selectively injured endothelium results in pulmonary vessels contraction and relaxation, which eventually leads to pulmonary hypertension (Imai et al., 2005; Price et al., 2012).

Neutrophil migration is common in barrier dysfunction and they move along the endothelium before emigrating into the interstitium and alveolar space. Selectin-mediated rolling of neutrophils and integrin-mediated arrest on the endothelium allows the neutrophil to extravasate through inter-endothelial junctions (Burns et al., 2003). The intensity of neutrophil penetration is based on the chemokines and cytokines released at the primary sites of inflammation (Grommes and Soehnlein, 2010).

During LPS-induced ALI, neutrophil infiltration into the alveolar lumen and interstitium and escalated numbers of macrophages in the thickened septa are the primary diagnostic histolopathologic abnormalities compared to lungs of normal animals with thin septal walls, occasional macrophages, and low number of neutrophils (Rittirsch et al., 2008). Ultrafine carbon particles, which are major components of air pollutants, induced lung injury in mice and resulted in increased disruption of blood- gas barrier. Lungs treated with LPS and ultrafine carbon particles showed significant swelling, fragmentation and denuded basement membrane. Electron microscopy images showed the presence of ultrafine carbon particles in denuded basement membrane indicating their role in disintegration of basement membrane and establishment of pulmonary edema and swelling. Transport of ultrafine carbon particles from the blood-air barrier to the alveolar

lumen through denuded basement membrane, which in turn leads to acute lung injury (Inoue et al., 2009).

Terminal ALI patients have lung histopathologic lesions characterized by progressive fibro-proliferative reactions by mesenchymal cell migration into the air spaces. Regulatory signals required for the migration is a set of proteins expressed on the air space area, which are exhibiting homology to PDGF. The process of fibro-proliferation is anatomically identical to the granulation reaction seen during wound healing (Snyder et al., 1991).

Resolution of inflammation requires the effective removal of accumulated edema, phagocytosis of activated/dead inflammatory cells, and functional restoration of endothelial and epithelial barriers (Matthay et al., 2012). In addition, transforming growth factor (TGF)-β, produced by CD4+ CD25+ Tregs, influences the apoptosis of activated neutrophils at the site of injury and inhibits pro-inflammatory cytokine production by macrophages (DeAlessio et al., 2009).

1.2.2 Pneumonia

Pneumonia caused by bacterial, viral or fungal infections results in alveolar inflammation characterized by accumulation of cellular and inflammatory debris in the alveoli leading to clinical symptoms such as cough with phlegm, fever, and difficult breathing. Influenza A and respiratory syncytial virus are major contributors of viral pneumonia in humans (Marcos et al., 2009). Early data from the 1920s revealed increased mortality and morbidity due to bacterial infection in influenza patients (Metersky et al., 2012).

Streptococcus pneumoniae is a major causative agent for lower respiratory tract infection and subsequent bacterial pneumonia in children infected with Influenza virus. A significant portion of viral-bacterial co-pathogenesis could be prevented by bacterial vaccines (Madhi et al., 2004). *S. pneumoniae* cells encounter mucus secretions of nasal cavity immediately after the infection. Outer capsule of pneumococcus minimizes bacterial entrapment in the nasal mucosa, thereby allowing the pneumococci to access the epithelial layer. Immediately after infection, cells invaded by pneumococcal organisms are found within the glycocalyx layer of the respiratory and olfactory epithelium (Nelson et al., 2006).

In *S. pneumoniae* infection, the TLRs essentially detect numerous PAMPs and induce an active innate immune response against the pathogen. *S. pneumoniae* infection triggers the innate immune system through both TLR2-dependent and independent pathways. TLR2 has been considered to regulate innate immune response to *S. pneumoniae* infection, through interaction with lipoteichoic acid, peptidoglycan, and the lipoproteins, which are elements found within bacterial cell wall. This recognition by TLR2 is critical activate the immune system to contain *S. pneumoniae* infection (Lee et al., 2007). Vos et al. reported that TLR2 is important in enhancing phagocytosis and oxidative killing of the *S. pneumonia* by the granulocytes (de Vos et al., 2015). TLR4 also has a role in body's innate immunity against *S. pneumoniae* infection. Recognition and interaction between the TLR4 and the pneumomolysin causes inhibition of growth of *S. pneumoniae*, especially in the nasopharynx colonization (Wilson et al., 2014).

Depletion of alveolar macrophages in murine pneumonia model contributed to increased lung inflammation and inadequate clearance of apoptotic neutrophils (Knapp et

al., 2003). Additionally, depletion of alveolar macrophages led to a high occurrence of mortality due to the accumulation of necrotic or apoptotic cells in lung (Barnes, 2004). *S. pneumoniae* is detected by alveolar macrophage and can be internalized and processed to phagosomes, which progressively become phago-lysosomes. In immune-deficient individuals, phagocytosis of *S. pneumoniae* by alveolar macrophages is likely less effective than it is in immune competent individuals. But complement cascade-mediated *S. pneumoniae* phagocytosis and efficient processing to phago-lysosomes is reported in individuals with antibodies against the bacteria (Gordon et al., 2000).

1.3 Innate immune system

The innate immune system is the first line of defense in detecting and responding towards a pathogen invasion. It is composed of pathogen-associated molecular pattern-recognizing receptors such as TLRs, NLRs and RIGs, and immune cells such as neutrophils and macrophages. Innate immune receptors not only detect the specific molecular patterns, but also trigger phagocytosis and activation of inflammatory pathways (Janeway and Medzhitov, 2002). The immune system is comprised of various immune cells such as neutrophils, macrophages, mast cells, natural killer (NK) cells, and dendritic cells (DCs). Two cell types and their role in innate immunity, which are the focus of this thesis, are described below.

1.3.1 Neutrophils in innate immune system

Neutrophils are innate immune cells that provide resistance for the host against various threats to the body, including microbial pathogens. Neutrophil precursors are produced and differentiate in the bone marrow. Once neutrophils mature, they are

exported to the blood stream and are circulated until they undergo apoptosis and are removed from circulation by macrophages in the bone marrow, spleen, and liver (Mathias et al., 2006).

In response to inflammatory stimuli, the neutrophils migrate towards the infected tissues and control the invading pathogens by producing proteolytic enzymes, antimicrobial peptides, and reactive oxygen species (Brinkmann et al., 2004). Neutrophils have both the oxygen-dependent and independent mechanisms to eliminate pathogens. The oxygen-independent mechanism involves the release of antimicrobial proteins and peptides into the phagosomes (Lehrer and Ganz, 1999). Oxygen-dependent mechanisms involve the production and release of potent antimicrobial free radicals produced by NADPH oxidase and myeloperoxidases (Serhan and Chiang, 2004). Neutrophils can release granule proteins and chromatin that forms an extracellular trap called neutrophil extracellular traps (NETs), capable of trapping and degrading virulence factors in bacteria (Brinkmann et al., 2004).

Phagosomal membrane translocation of cytoplasmic oxidative enzymes during the neutrophil activation leads to the activation of NADPH oxidase, which in turn produces antimicrobial products such as superoxide. Activation of myeloperoxidase (MPO) catalyzes the production of hypochlorous acid and other secondarily derived reactive oxygen species (ROS) such as singlet oxygen and hydroxyl radicals. After the activation and internalization of the pathogen, neutrophils undergo spontaneous apoptosis in order to bring the immune system to homeostasis. Neutrophil apoptosis lead to the resolution of immune response (Amulic et al., 2012; Douda et al., 2014).

1.3.1.1 Neutrophil chemotaxis

Chemotaxis, the guided movement of cells by chemical gradients created by chemoattractant proteins, such as chemokines and bacterial products produced at the site of inflammation, probably emerged early in eukaryotic evolution (Kay et al., 2008). Neutrophils are highly mobile cells that readily undergo chemotaxis. Neutrophils can detect as low as 1/100th of a chemokine gradient. They polarize their migration towards bacterial peptides over a pool of other chemical gradients to reach sites of inflammation. Neutrophils adopt a 'pseudopodia- centered model' to migrate towards the chemokine concentration gradient. Neutrophils undergo amoeboid-type movement, the most primitive form of single cell migration. Amoeboid-type of cell migration is characterized by rapid morphological changes with minimal substrate contact by the cell followed by high velocity migration (often 5-28μm/min) (Lammermann and Sixt, 2009). Neutrophil migration is mainly orchestrated by polymerization and depolymerisation of actin proteins (Arraes et al., 2006).

Chemokines are cationic molecules that immobilize on endothelial surface with the help of anionic heparin sulphates. Chemokine distribution is concentration-dependent and, eventually, creates an intravascular chemokine gradient for neutrophil chemotaxis. Neutrophil chemotaxis begins with the phosphorylation and activation of specific G-protein coupled chemokine receptors on the neutrophil surface, which will result in a conformational change of surface integrin proteins. Binding between lymphocyte associated antigen (LFA) on neutrophil surface and intercellular adhesion molecules of endothelium induces a conformational change to talin-1, a cytoskeletal protein, leading to firm attachment and slow rolling of neutrophils (Wang et al., 2011).

Neutrophil recruitment to the site of inflammation follows specific steps: tethering, rolling, adhesion, crawling, and transmigration. The process of neutrophil chemotaxis is initiated by changes on the endothelial surface caused by pathogen-induced or tissue injury-mediated secretion of specific chemokines that are released from tissue-resident innate immune cells (Kolaczkowska and Kubes, 2013). Endothelial cells can also be activated directly through their surface receptors recognizing specific pathogen associated patterns (PAMPs) or damage associated molecular patterns (DAMPs). Once activated, endothelial cells express specific adhesion molecules like P- and E-selectin on their surface. These selectin proteins play a crucial role in initiation and maximization of neutrophil recruitment to the site of injury (Sundd et al., 2011). Once expressed on the endothelial surface, selectins bind to their definitive ligands, such as P-selectin glycoprotein ligand 1, on the neutrophil surface leading to the slowing down of free moving neutrophils. L- selectin plays key role in accelerating the secondary tethering of neutrophils (McDonald and Kubes, 2011; Sundd et al., 2013). Eventually, neutrophils attach to endothelium while experiencing a shear stress of 10-100$\mu N/cm^2$ (Halilovic et al., 2015). This rapid deceleration requires extensive manipulation of adhesive bonds between neutrophils and endothelial surface. The surface of neutrophils is covered with LFA, which binds to ICAM1 and ICAM2 on vascular endothelial membranes to allow the neutrophil to be tethered to the endothelium (Sanz and Kubes, 2012; Halilovic et al., 2015).

Activated and adhered neutrophils exhibit ‘pirouetting’ behavior as they begin to elongate pseudopodia and scan nearby areas while adhered to a location with the endothelium. During the adhesion stage, neutrophils prepare for transmigration by

crawling to endothelial cell junctions. The mechanotactic components of neutrophil crawling over the endothelium are orchestrated by many signals includes VAV1, a Rho family GTPase, and CDC42, a cell division regulator and regulator of actin during neutrophil polarization and migration. Actin binding protein, known as debrin-like protein (DLB), in association with β2 integrin proteins, induces conformational changes in the actin cytoskeleton of neutrophils (Futosi et al., 2013).

Neutrophil transmigration from blood vessels to the injury site takes approximately 15- 20 min (2-5 min to cross the endothelium and 5-15 min to cross the basement membrane) (Muller, 2013). Along with ICAMs and integrins, neutrophils require adhesion molecules such as vascular cell adhesion protein-1, platelet/endothelial cell adhesion molecule 1, CD99, vascular adhesion protein 1, and leukocyte specific protein 1 (LSP1) to cross the endothelial barrier (Li Jeon et al., 2002). Neutrophils migrate to the site of injury either between two endothelial cells (paracellular migration) or through an endothelial cell (transcellular migration). Paracellular migration requires relaxation of endothelial cell junctions and is the most common mode of neutrophil migration. During transcellular migration, endothelial cells form 'domes' around adhered neutrophils by extending villi-like projections (Parkos et al., 1992). Adhesion molecules such as ICAM1 and VCAM1 are abundant in domes, which actively interact with LFA-1 and integrin α4 of neutrophils. LSP1, an actin binding protein expressed abundantly in endothelial cytoplasm and nucleus, regulates endothelial cell dome formation. This process is completely different from classical endocytosis because neutrophils never merge with the intracellular compartment of endothelium (Ley et al., 2007; Halilovic et al., 2015).

During the initiation of paracellular migration, cytoskeleton reorganization in endothelial cells enables them to alter their attachment of the extracellular matrix (ECM) via specific focal adhesions (Okun et al., 2009). The endothelial basement membrane is composed of a complex mesh-like ECM composed of proteins, such as laminin and collagen. Neutrophils release proteases, such as MMPs and neutrophil elastase, to degrade ECM in order to 'cut' its way to the site of injury (Marki et al., 2015). Extracellular signal regulated kinases (ERK) and p38 are two MAP Kinase proteins playing crucial role in PMNs chemotaxis towards fMLP. Neutrophil movement towards fMLP gradient regulates via specific G protein coupled receptor, known as formyl peptide receptor (FPR). The fMLP-induced ERK regulated activity of G protein coupled receptor kinase 2 (GRK2) and inhibited neutrophil chemotaxis at escalated concentrations, whereas p38 acted as a non-canonical G protein coupled receptor kinase that phosphorylated FPR itself and activate neutrophil migration by arresting GRK2 pathway at reduced concentrations (Arraes et al., 2006).

1.3.1.2 Neutrophil apoptosis

There are specific receptors, known as "death receptors" which can induce apoptosis. These receptors are members of the tumor necrosis factor or nerve growth factor receptor super family or the Fas ligand receptors (Kennedy and DeLeo, 2008). Activation of death receptors results in initiation of the extrinsic pathway of apoptosis. However, Fecho and colleagues proposed that neutrophil apoptosis from Fas receptor or Fas ligand deficient mice appears to be normal, suggesting that extrinsic activation is unimportant in activating neutrophil apoptosis (Fecho and Cohen, 1998). Instead,

neutrophil apoptosis may be due to the internal signaling that activates the intrinsic pathway of apoptosis or another extrinsic signaling pathway that has not been identified. Spontaneous neutrophil apoptosis via the intrinsic pathway involves a change in balance among the members of the Bcl-2 family of proteins (Luo and Loison, 2008). Mcl-1 and A1 concentrations are upregulated by GM-CSF or LPS stimulation, inducing neutrophil survival. There are several distal proteins, for example caspase 3, caspase 8, and caspase 9, that trigger proteolytic cascades that lead to the neutrophil apoptosis (Scheel-Toellner et al., 2004).

There is some evidence suggesting neutrophil apoptosis through the extrinsic pathway of apoptosis. Fas is one of the important receptors that forms death-inducing signaling complex (DISC) and is expressed on the surface of neutrophils, as well as on virtually all other mammalian cells. Fas-associated death domain (FADD) is an intracellular domain through which Fas leads to accumulation of death domains on the cytoplasmic side of the cell membrane, which leads to the induction of caspase pathway and apoptosis (Scheel-Toellner et al., 2004). TNF alpha-receptor, a transmembrane protein and a TNFR-associated death domain (TRADD) in the cytoplasmic region of the protein, also regulates apoptosis of neutrophils. It has been shown that intercepting TNFR activity using antibodies inhibits neutrophil apoptosis (Gon et al., 1996). Bianchi and colleagues shown that TNF-related apoptosis inducing ligand (TRAIL) also induces apoptosis in neutrophils. There are five TRAIL receptors (TRAIL-R1, TRAIL- R2, TRAIL-R3, TRAIL-R4, and TRAIL-R5) that have been identified on neutrophil surfaces (Bianchi et al., 2006).

1.3.2 Macrophages in innate immunity

Mononuclear phagocytic cells, such as macrophages and monocytes, are involved in neutralizing pathogens and maintaining tissue homeostasis by directing the inflammatory process, both through activation and resolution. In mammals, monocytes contribute 4-10% of the total nucleated blood cell population and have a half-life in circulation of 20hr (Cavaillon, 1994). Monocytes originate in primary lymphoid organs, such as the bone marrow, from myeloid progenitor cells. Alveolar macrophages (AMs) are resident macrophage cells located within the alveoli of the lung. They participate in pathogen recognition through PRRs, such as TLRs, NODs, and RIGs. Upon activation, AMs secrete pro-inflammatory cytokines such as IL-1β, TNF-α, IL-2, which in turn activate alveolar epithelial cells and endothelial cells and recruit of neutrophils (Herold et al., 2011; Balhara and Gounni, 2012). Additional monocytes are recruited to the lung in response to the release of monocyte chemotactic protein-1 (MCP-1) from pulmonary and inflammatory cells (Lee et al., 2008).

When alveolar macrophages are depleted in mice infected with pneumococcus, inefficient clearance of necrotic and apoptotic neutrophils was observed and mice exhibited higher mortality compared to controls (Boyd et al., 2012). During LPS exposure, another macrophage population involved in the resulting inflammatory response in some species is pulmonary intravascular macrophages (PIMs). According to the functional analysis of PIMs vs AMs, PIMs are more aggressively phagocytic and cytotoxic than PAMs but are comparable in antibody-dependent cellular cytotoxicity. Analysis of effector cells showed that PIMs are less efficient or equal in cancer cell-lysis compared to PAMs (Chitko-McKown et al., 1991; Thanawongnuwech, 1998). Macrophages respond to the specific “find me” signals for example specific nucleotides

released though channels like pannexin 1 channels from apoptotic neutrophils. Soluble proteins such as pentraxins, ficolins, collectins secreted by innate cells and are collectively referred as bridging molecules since they involve in the molecular pattern recognition and response (Herold et al., 2011).

In general, macrophages are classified into two distinct subsets, namely M1 and M2. M1 macrophage subset is involved in killing or phagocytic functions, whereas the M2 subset is involved in healing or growth functions (Italiani and Boraschi, 2014). Based on the type of initial stimulus, macrophages use T cells to amplify their response effects along with polarizing into a M1 or M2 phenotype to generate a specific response. It is believed that macrophage-directed adaptive immune responses will be either M1/Th1 or M2/Th2. Because of immunological redundancy, innate immune system converts M1 to M2 or vice versa based on the requirement (Covarrubias et al., 2013). This inter-plasticity of M1 and M2 macrophage subsets are usually analyzed by recombinant Cre-lox system under iNOS or arginase promoters (Cassol et al., 2009).

1.4 Toll-like receptors

Microorganisms continuously challenge the host defense, which includes the innate, and the acquired immune systems with the innate system acting as the first line of immune defense (Akira and Takeda, 2004). The notion that there may be specific receptors for microbial molecules led to the identification of a group of proteins called Toll-like receptors (TLRs). TLRs are a family of evolutionarily conserved pathogen recognition receptors that play a critical role in innate immunity and in early response against invading pathogens (Akira et al., 2001). The protein Toll was first reported as a receptor involved in embryonic development of the fruit fly (Hashimoto et al., 1988) and

later, TLRs, designated as Toll's mammalian homologues, were described (Medzhitov et al., 1997). TLR function was rapidly determined to be responsible for recognition of microbial invaders (Basu and Fenton, 2004). Thirteen TLRs (named TLR1 to TLR13) have been identified in humans and mice, and equivalent forms of many of these have been identified in other mammalian species. TLRs recognize extracellular and intracellular ligands consisting of a variety of microbial proteins and peptides, polysaccharides, proteoglycan, nucleic acids, and phospholipids (Liu et al., 2010).

1.4.1 Ligands of TLRs

Each TLR recognizes specific PAMPs derived from various microorganisms including bacteria, viruses, protozoa and fungi (Akira et al., 2006). In addition to microbial PAMPs, an escalating number of endogenous molecules are reported as candidate ligands of TLRs (Chiron et al., 2008). The main endogenous molecules that activate TLRs are fibronectin, heparin sulphate, biglycan, fibrinogen, oligosaccharides of hyaluronan, and hyaluronan breakdown fragments (Ian R. Rifkin, 2005; Yu et al., 2010). In pathological conditions, these endogenous molecules are secreted by inflamed/dying cell or by activated cells in a non-conventional lysosomal-dependent manner (Pollanen et al., 2009). These endogenous ligands act as alarms and give early warning signals to innate and adaptive immune systems (Liu et al., 2010).

TLR2 and TLR4 are the principal receptors involved in the recognition of various bacterial cell wall components. TLR4 is crucial for effective responses to LPS, a component of Gram-negative bacterial cell walls (Hong-Geller et al., 2008). Recognition of LPS, part of bacterial cell membrane, by TLR4 requires a concerted effort by additional molecules including LBP (LPS Binding Protein), CD14, and MD2. Integrin

CD11b/CD18 also play a role in TLR signaling towards LPS stimulus and these molecules provide negative feedback mechanism to limit the TLR action during long-term LPS signals (Han et al., 2010). TLRs3, 7, and 8 play important roles in responses to viruses. Double-stranded viral RNA activates TLR3 while TLRs7 and 8 are activated by single-stranded RNAs (Albrecht et al., 2004). TLR9 plays an important role in detection of CpG DNA present in bacteria and viruses (Lund et al., 2003). Bacterial flagellin proteins are recognized by TLR5. TLR1 is responsible for the detection of triacyl lipopeptides (Aboussahoud et al., 2010).

1.4.2 TLR signaling

TLR signaling is initiated by dimerization of TLRs, which can form homodimers (such as TLR4) or hetero-dimers (such as TLR2 and TLR1). Other than TLR3, all the TLRs using MyD88 as primary adaptor molecule to initiate the signaling cascade (Akira and Takeda, 2004). After ligand binding, TLRs/IL-1Rs domain, dimerize and undergo the conformational change required for the recruitment of downstream signaling molecules. These include the adaptor molecules myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6). The TLR signaling pathways follow two types of downstream activation, MyD88-dependent and MyD88-independent pathways, which eventually switch on the pro-inflammatory cytokine response through NF-κB and AP1 up-regulating nuclear transcription (Akira, 2003). TLR3 utilizes a TIR-domain-containing adapter-inducing interferon (TRIF) dependent pathway that ultimately ends in up-regulation of interferon pathway. The activation of TRIF TLR4 utilizes TRIF-

related adaptor molecule (TRAM) as a “bridge molecule” to activate TRIF. The activation of IRF by TLRs via MyD88 or TRIF leads to the production of type I interferon (Sabroe et al., 2008).

The stimulus induced dimerization of the receptors’ extracellular domains leads to coordinated conformational changes that, in turn, lead to self-association or reorganization of the cytoplasmic domain- TIRs, thereby creating a new molecular docking site for the recruitment of signaling adaptor molecules. In the model of the TLR4 homodimer proposed by Miguel and colleagues, the interface has significant interactions involving the BB loops, a loop which connects second β-strand and a second helix includes a proline residue, of the two subunits. Proline residues give the normal and rigid conformation of the BB loop and the replacement or substitution of these residues will result in the internal homodimer geometry distortion (Miguel et al., 2007). The TIRs offer a rotational flexibility to the linkers with the 2- fold axis symmetry of the receptors. TLR4 homodimer forms a flat, slightly curved architecture to make a top or membrane proximal surface of the structure (Deng et al., 2013). Upon ligand activation, TLR2 can form heteromers with TLR1 or TLR6 (Farhat et al., 2008).

1.4.3 TLR cellular localization

TLRs actively localize to different cytoplasmic compartments depending on their functional properties. TLR4, one of the five human TLRs showing orthology with Toll of Drosophila, is a membrane-spanning protein that binds with LPS. TLR4 is normally localized to plasma membrane and Golgi complex in resting human monocytes (Shi and Kehrl, 2010). It is associated with CD14 and MD2 protein, which help in the translocation of TLR4 to the cell surface (Togbe et al., 2007). The presence of TLR4 has

been observed in the cytoplasm and nucleus of pulmonary intravascular macrophages and septal endothelial cells in normal and inflamed cells (Schneberger et al., 2010). It has been proposed that the whole complex of TLR4-CD14-MD2 cycles to Golgi complex and recycles rapidly (Latz et al., 2002). The internalization process of TLR4-LPS complex is observed within 15 min after ligand activation and is mediated by clathrin and dynamin. Endosomal internalization of TLR4 complex is considered to be one of the negative regulatory pathways to limit TLR4 signaling and an ubiquitination step leads to degradation of the TLR4 (Palsson-McDermott et al., 2009). TLR4 is localized in the Golgi complex and not on the plasma membrane in m-ICc12 cell line (Mathias W. Hornef et al., 2002).

On the other hand, TLR2 has been identified in early and late endosomes, but not in the Golgi complex or endoplasmic reticulum. TLR2 forms a heterodimer along with TLR1/6. CD14 and CD36 are the co-receptors of the TLR2- TLR1/6 heterodimer complex. Activation of type 1 IFN pathway, but not TNF-α production, demands the internalization of TLR2. This demonstrates that TLR2 can activate two different signaling pathways from two different locations (Barbalat et al., 2009).

TLR3, 7, and 9 have specific retention signals that localize them to the Golgi complex and prevent translocation to cell membrane to prevent inadvertent activation of intracellular TLRs (especially TLR9) by nucleic acids of self-origin. For TLR9, either its transmembrane region or transmembrane sequence is efficient for its retention in the Golig comprex (Leifer et al., 2006). The intracellular TLRs move to endosomes after activation by their respective ligands (Barton et al., 2005). Taken together, there is

diversity in the localization of TLRs in various cells, but further works needs to be performed to further evaluate TLR expression and localization in all cell types.

1.4.4 Cross expression of TLRs

Though each TLR has a highly specific ability to recognize a particular microbial pattern, recent papers suggest that some ligands are able to activate the expression of TLRs in addition to their traditional assigned specific receptor. Elevated TLR mRNA and protein expression in response to stimulation with cytokines, pathogenic organisms, and cases of mucosal inflammation have been reported (Fan, 2009). Cells treated with TLR4 ligands showed increased expression of TLR5, 7 and 9 in STC-1 and RAW 264.7 (Ghosh et al., 2007). Similarly increased expression of TLR2 and TLR4 occurred in the presence of PAMPs other than LPS on submucosal macrophages in inflamed mucosa. In the presence of Pam3CSK4, which is the ligand of TLR1/2, TLR5 was found to be strongly upregulated (Ozinsky et al., 2000). Later studies proved that the presence of Pam3CSK4 stimulates the binding of TLR2 with TLR1 and influences TLR5 up-regulation (Barbara Koller, 2008). We have previously shown that TLR2 and TLR9 expression is increased in the lungs of horses following intravenous exposure to *E. coli* LPS, which is a ligand for TLR4 (Singh Suri et al., 2006; Schneberger et al., 2011). Ligands of TLR2 or 4, in combination with TLR7/8, up-regulate the expression of IFN-γ, IL-12, and IFN-α expression in human peripheral blood mononuclear cells (Ghosh et al., 2007). There is a great need for clarity on the expression of individual TLRs following the ligation of another single TLR or simultaneous ligation of multiple TLRs by their respective ligands.

1.4.5 Toll-like receptor 10

TLR10 is recently identified TLR whose ligand and signaling pathway is still unknown. It is expressed in a variety of organs including lymph nodes, spleen, thymus, and lungs (Chuang and Ulevitch, 2001). Functional TLR10 is absent in mice and no model organisms have been identified thus far. Studies in chickens confirm the presence of ten TLRs (TLR1- 10) with gene expression patterns being homologous to mammalian systems (Chuang and Ulevitch, 2001).

1.4.5.1 Structure

Human TLR10 is the most recently identified TLR protein. It encodes 811 amino acids and has a molecular mass 95 kDa. *TLR10* is a 3269 bases gene in chromosome 4 and the protein consists of a conventional leucine cysteine rich domain and a cytoplasmic toll interleukin-1 receptor domain. TLR10 shows homology with other TLRs in their structure with a signaling peptide (1-19 amino acids), which leads to protein for its membrane translocation. The location of the cysteine residues within the amino acid is at 526, 528, 553, and 574 (Chuang and Ulevitch, 2001). TLR10 exhibits 50% homology with TLR1, 49% with TLR6, and 30% with TLR2. A 13 amino acid signaling sequence located in the C-terminal of TLR2, which is involved in TLR2 down-regulation, is also present in TLR10. Because of this homology, TLR10 may follow a similar type of downstream signaling after activation (Lazarus et al., 2004). Guan et al proposed triacyl lipopeptides mediated activation and dimerization of TLR10 with TLR2 through an alternative pathway of TLR2 activation (Guan et al., 2010).

1.4.5.2 TLR10: Evolutionary aspects

Sequence analysis and phylogenetic mapping have grouped vertebrate TLRs into six 'gene families'- namely TLR1, TLR3, TLR4, TLR5, TLR7, and TLR11. Generally in vertebrates, each TLR family is encoded in a single gene but the TLR1 super family shows exception with TLR1, TLR2, TLR6, and TLR10 being encoded by multiple genes. This gene cluster stably evolved and is conserved by high positive purifying selection (Calvano et al., 2005). Two evolutionarily conserved forms of TLR5 in rainbow trout show evolutionary analogy of mammalian TLRs (Rebl et al., 2010).

Independent gene duplication was observed in the TLR1 family, both in avian and mammalian systems. Gene conversion of the human TLR1 family occurred 42-44 million years ago in the common primate ancestor of human, chimpanzee, orangutan, and rhesus monkey. There are two orthologous groups of TLR1 family that were present in mammals and avian as TLR1A/TLR10 and TLR1B/TLR1/TLR6, which might be the product of gene duplication that occurred about 360 million years ago. Computational modeling of the TLR10/TLR2 heterodimer indicates the presence of a lipopeptide binding pocket, which shows homology to the binding sites in the TLR1/TLR2 complex (Huang et al., 2011).

1.4.5.3 TLR10 in Cell Signaling

Hypoxia induces the up-regulation of TLR10 in a human monocyte cell line, THP-1 (Kim et al., 2010). The hypoxic condition in this model was designed to mimic low oxygen conditions that occur during bacterial infection or tissue injury. Systemic hypoxia induces production of reactive oxygen species (ROS), which up-regulates the

pro-inflammatory transcription factors, such as NF-κB and AP-1, which in turn up-regulate TLR10. Luciferase binding assays indicate a potential NF-κB binding site in TLR10 gene sequence (Kim et al., 2010). TLR10 mRNA expression was found in early B cell development and its translation was mainly observed during B cell differentiation (Bourke et al., 2003). TLR10 dimerization was predicted to occur during its activation with the predictions ranging from 100% for a TLR10 homodimer, 87% for a TLR1 complex, and 80% for a complex with TLR1/2. Accordingly, TLR10 is suspected to signal through the MyD88-dependent pathway to initiate the NF-κB nuclear translocation (Hasan et al., 2005). Increased expression of TLR10 and subsequent activation of interferon 1 signaling when dendritic cells were challenged with viral agents indicates the probable ligand for TLR10 may be viral in origin (Nyman et al., 2008). Co-localization of TLR10 with TLR2 in early phagosomes of activated macrophages suggests that TLR10 may use TLR2 as its co-receptor for its activation and signaling. Extracellular domains of TLR10 and TLR2 were found to be physically interacting with each other in a ligand dependent manner (Guan et al., 2010).

1.4.5.4 TLR10 polymorphisms and disease susceptibility

Genetic variations in TLR10 gene may play a critical role in human asthma, as there were 78 single nucleotide polymorphisms of the TLR10 gene discovered in 47 samples from asthmatics of comprised of multiple USA ethnic groups. The single nucleotide polymorphism (SNP) frequency was five in the European American group and twenty-three in the African American ethnic group. It is also proposed that the association of these two independent samples and phenotypes provides high susceptibility

for asthma (Lazarus et al., 2004; Tizaoui et al., 2015). Since TLR6 and 10 are found on same chromosome with a 58kb distance apart, it is difficult to differentiate the independent role of TLR6 and TLR10 polymorphism in asthmatics using current molecular marker techniques. Two polymorphisms in TLR6 gene and seven in TLR10 gene were mapped from 322 patients suffering from clinical asthma (Lazarus et al., 2004). TLR10 polymorphisms have also been identified in patients with nasopharyngeal carcinoma patients, demonstrating a potential role of TLR10 in cancer biology. TLR10 polymorphism has been observed in prostate cancer patients. Chen et al suggested that the TLR1-TL6-TLR10 gene cluster polymorphism might increase the risk of developing prostate cancer (Chen et al., 2007).

Out of ten TLRs present in the humans, TLR10 has been recently identified and is the only "orphan" receptor, with no confirmed ligand or signaling pathway. Studies have shown that variation in the TLR10 gene might affect the occurrence of disease conditions such as asthma, nasopharyngeal carcinoma, and prostate cancer. The presence of TLR10 in immune cells such as macrophages and neutrophils indicates a potential, but unknown, role in the detection of PAMPs and activation of innate immunity.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

2.1 Hypotheses

1. TLR10 is expressed in the lungs and its expression is altered during inflammation.
2. TLR10 is expressed in human neutrophils and LPS alters its spatial-temporal expression.
3. TLR10 plays a role in neutrophil chemotaxis towards fMLP.
4. TLR10 is involved in macrophage immune response towards *S. pneumoniae* infection.

2.2 Objectives

1. To characterize and compare the expression profiles of TLR10 in normal and inflamed lung tissues of cattle, chicken, pig, rat and dog
2. To characterize the expression and localization of TLR10 in control and LPS activated human neutrophils
3. To study the role of TLR10 in neutrophil chemotaxis
4. To study TLR10 expression in human macrophages
5. To characterize the role of TLR10 in *S. pneumoniae* infection in human macrophages

2.3 Rationale

Lungs are the largest and significant component of respiratory system with both respiratory and non-respiratory functions. Lungs are the sites for gas exchange and

involved in key physiological functions. Since lungs continuously interact with outer environment, they are the major sites of bacterial, viral or fungal infections leading to acute and chronic inflammatory diseases (Marsland and Gollwitzer, 2014). The diseases of respiratory system affect millions of people and livestock around the world, and cause significant mortality, morbidity and economic losses. Apart from infectious agents, physical agents such as cigarette smoke, environmental pollution, as well as genetic factors contributes to lung inflammation (MacNee, 2005).

Infectious agents express highly conserved molecular patterns, which are recognized by innate immune receptors such as TLRs of the host immune system. Activation of innate immune receptors alerts the immune system to induce proinflammatory responses such as cytokines secretion, and activation of immune cells such as neutrophils and macrophages (Akira et al., 2006). There are a total of 13 TLRs that recognize specific ligands associated with various pathogens. TLR10 is the recently identified TLR family protein and to date, there are no data available about the ligand, signaling pathway and role in inflammation. Hence, it is refereed to as an "orphan receptor" (Janeway and Medzhitov, 2002).

Recent data on the roles of TLR10 in immune response to viral and bacterial infection points towards its functional importance. Genetic polymorphisms in TLR10 gene make individuals susceptible to chronic inflammatory diseases including asthma, and COPD (Tizaoui et al., 2015). Currently, there are no data on the cell specific expression of TLR10 in the lung. Because the inflammatory processes are driven through coordinated actions of a variety of cells, it is important to understand the *in situ* expression of TLR10 in mammalian lungs. Because of the role of neutrophils as primary

effector cells in acute inflammation, I propose to examine the expression of TLR10 in normal and activated neutrophils, and design experiments to understand the role of TLR10 in neutrophil biology. Furthermore, the role of TLR10 in handling of bacteria such as *S. pneumoniae* is not understood. Therefore, I designed experiments to obtain data to fill gaps in our knowledge related to the biology of TLR10.

CHAPTER 3: TOLL-LIKE RECEPTOR 10 EXPRESSION IN CHICKEN, CATTLE, PIG, DOG, AND RAT LUNGS

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Running title: TLR10 expression in multiple species

Keywords: vascular endothelium, neutrophils, inflammation, pulmonary intravascular macrophages, immuno-electron microscopy

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3.1 Abstract

Toll-like receptors (TLRs) are conserved immune receptors that play critical roles in innate immunity and are known as “gate keepers” of immune system. TLR10 is identified as type 1 plasma membrane protein but the identity of its ligand remains unclear. Till date, no data are available on tissue and cell specific expression of TLR10 in normal and inflamed lungs of domestic animal species, and rat, which is commonly used as a model to study human diseases. We characterized a commercially available TLR10 antibody for use in cattle, pig, dog, chicken, and rat. Western blotting of total lung protein extracts from cattle, dog, pig and chicken showed a band of 95kDa, which is similar to the molecular weight of TLR10. The immuno-histochemical and immuno-electron microscopic data show TLR10 expression in vascular endothelium and smooth muscles in lungs of control and inflamed animals. Further, we found that expression of TLR10 in bovine neutrophils is altered upon treatment with *E. coli* lipopolysaccharide. These data show TLR10 expression in the lungs of these mammalian species and that activation of bovine neutrophils alters the expression of TLR10.

3.2 Introduction

Toll-like receptors are members of the innate immune system and recognize pathogen associated molecular patterns (Takeda and Akira, 2004). Since the identification of Toll proteins in Drosophila, more than 10 TLR proteins have been identified in mouse and humans (Anderson et al., 1985; Hashimoto et al., 1988). These TLRs recognize microbial molecules such as lipopolysaccharides and initiate cell signals to activate immune cells and initiate inflammation. The functions of some of the TLRs such as TLR4, TLR2, and TLR9 have been studied extensively and point to their critical roles in organ pathophysiology (Andonegui et al., 2003; Dubourdeau et al., 2006). However, the data on some of the TLRs including TLR10 are scarce.

TLR10 is expressed in immune cells such as neutrophils, macrophages, dendritic cells, organs such as spleen, thymus, and at lower levels in lungs of humans (Chuang and Ulevitch, 2001). The expression of TLR10 mRNA was reported in early B cell development and the translation commitment mainly observed during B cell differentiation (Bourke et al., 2003). While TLR10 is nonfunctional in mice due to several retroviral insertions, it is functional in avian system (Brownlie and Allan, 2010). Although the role of reactive oxygen species induced during hypoxia in upregulation of TLR10 mRNA in THP-1 cell line has been shown recently (Kim et al., 2010), there is yet no firm information on the identity of the ligand of TLR10.

Domestic animals are susceptible to many bacterial lung diseases and these respiratory diseases inflict heavy economic losses on animal industry through morbidity and mortality. In USA, *Mannheimia haemolytica* infections, despite development of vaccines and better management strategies, cause more than $1.1 billion economic loss to

cattle and sheep industry (Sathiamoorthy et al., 2011). In dogs, *Bordetella bronchiseptica* is a commonly occurring infection, and it causes lung disease (Goodnow, 1980). Pulmonary infections caused by pathogens such as Porcine Reproductive and Respiratory Syndrome Virus as well as *Haemophilus somnus* and Actinobacillus spp. cause significant mortality and morbidity in pigs (Chiers et al., 2002). Over the recent years, there have been advances in understanding the expression and biology of TLRs in domestic animal species. We have contributed data on the expression of TLR4 and TLR9 in normal and inflamed lungs of cattle, horses, pigs, and dogs (Wassef et al., 2004; Singh Suri et al., 2006; Schneberger et al., 2011). For example, TLR4 and TLR9 expression is altered in inflamed lungs of horses (Wassef et al., 2004). During these studies, we also found that pulmonary intravascular macrophages (PIMs), a population of macrophages unique to ruminants, equines and pigs, express TLR4 and TLR9 and their depletion leads to significant reduction in the amounts of TLR4 and TLR9 mRNA in lungs (Singh Suri et al., 2006; Schneberger et al., 2009). However, there are no data on the localization of TLR10 in lungs of domestic animal species.

Here we investigate the expression of TLR10 in lungs of pig, cattle, dog, rat, and chicken at tissue and subcellular levels using immuno-histochemistry, immuno-electron microscopy, confocal microscopy, and western blotting. We analyzed the changes in TLR10 expression on infection with *M. haemolytica* in cattle, Foal Adenovirus in chicken and H3N2 virus in pigs. Our data show that TLR10 is expressed in vascular endothelium and alveolar septa and the infection alters its expression in lungs of all the species.

3.3 Materials and methods

3.3.1 Animals

Research Ethics Committee of University of Saskatchewan approved all animals and protocols used in this experiment. Lung samples from chicken (n=8), cattle (n=5; 6 weeks old; male; Holstein-Friesian breed), pig (n=5; 6 week old male), dog and rat (n=5) were harvested, fixed 4% paraformaldehyde and embedded in paraffin for routine and immuno-histochemical studies. Male calves were infected intra-tracheally with *M. haemolytica* (n=7; 6 weeks old; Holstein-Friesian breed; total dose $20x10^9$ *M. haemolytica*/ calf) and pigs (n=6; 6 week old male) were infected with influenza A/ 98 (H3N2) TX98 strain (total dose of H3N2 ~10^5 pfu) and euthanized at 24hr after the infections. Chickens were challenged with *E. coli* (10^7 cfu/bird) and FAdV (10^4 $TCID_{50}$/bird; n=6 each) and euthanized 24hr post-infection. Lastly, rats (n=8) were challenged intratracheally with *E. coli* lipopolysaccharide (1.5 µg/kg) 18hr prior to euthanasia. Tissues were embedded in paraffin for light microscopy and in LR White for immuno-electron microscopy. Lung tissues collected in liquid nitrogen and frozen later were used for western blotting.

3.3.2 Western blotting

Frozen lung tissues were lysed and homogenized in 200µl freshly prepared lysis buffer composed of 150mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris (pH 8.0), 5mM ethylene-diamine-tetra-acetic acid (EDTA), and protease inhibitor cocktail (100µl/10ml; Sigma-Aldrich). Protein fraction collected were separated in 10% SDS-PAGE and transferred to Hybond-ECL nitrocellulose membrane (GE Healthcare, Mississauga, CA). Membrane was blocked for

nonspecific binding by 5% skim milk in 1X PBS for 1hr. The membranes were incubated with primary TLR10 antibody (1:600, 1mg/ml; Abcam, Cambridge, USA) for 12hr at 4°C followed by treatment with HRP conjugated anti-goat IgG secondary antibody (1:1000, Dako, Ontario, Canada), and color development (GE Healthcare Inc.). Blots were scanned digitally and unbiased contrast adjustments were done.

3.3.3 TLR10 immuno-histochemistry

Paraffin-embedded tissues from chicken, cattle, pig, dog, and rat were used for immunohistochemistry. Paraffin sections were deparaffinized and rehyderated before antigen retrieval with pepsin (2mg/ml) treatment for 45min and endogenous tissue peroxidase quenching with 0.5% hydrogen peroxide in methanol for 30min. The sections were incubated with 1% BSA in 1X PBS for one hour followed by exposure to TLR10 primary antibody (1:150, 6.67µg/ml) for one hour and HRP-conjugated anti-goat IgG (1:150) for 45min. The color development was carried out with a commercial color development kit (Vector Laboratories, Ontario, Canada). The controls included omission of primary antibody, incubation with isotype-matched antibody and the use of a peptide to block the binding of TLR10 antibody. We obtained custom-made 32 amino acid blocking peptide (Sequence: CHNRIQQLDLKTFEFNKELRYLDLSNNRLKSV) from New England Peptide (Gardner, MA, USA). Blocking peptide was dissolved in 1X PBS (pH 7.2; Concentration: 200µg/ml). We used 14µg/ml of the blocking peptide to block TLR10 polyclonal antibody. In brief, TLR10 antibody was diluted (1:150) in 1% BSA (in 1X PBS) and added the blocking peptide (final concentration: 14µg/ml). The primary antibody-blocking peptide mixture was incubated for 2hr at room temperature. Immuno-

histochemistry was performed, using this blocking peptide- antibody mixture, as explained above.

3.3.4 Immuno-gold electron microscopy

Lung tissues from cattle, dog, pig, and rat were embedded in LR White. Ultrathin (100nm) sections were prepared and placed on nickel grids. The sections were blocked with 1% serum albumin for 30min and incubated with TLR10 primary antibody (1:30) for one hour followed by exposure to gold-conjugated anti-goat secondary antibody (1:100; Fitzgerald Industries International, Concord, USA) for one hour. Negative control was performed by omitting primary antibody from the protocol.

3.3.5 Confocal microscopy

Isolated bovine neutrophils were incubated at 37°C and 5% CO_2 in RPMI-1640 with 10% fetal bovine serum and were treated with *E. coli* LPS (1μg/ml) for 60min. Neutrophils were fixed in 4% paraformaldehyde and permeabilized with 0.01% Triton X100. Nonspecific antigens were blocked by 5% BSA and Fcγ blocking was performed as previously described. Neutrophils were incubated with TLR10 antibody (1:250) one hour followed treatment with FITC-conjugated secondary antibody for 30min at room temperature. Cells were mounted in a medium containing DAPI and left overnight for proper conditioning of the slides for imaging. Confocal microscopy was performed in Leica TCS SP5 system (Leica Microsystems, Germany) with 63X oil immersion lens.

3.3.6 Statistical Analyses

Results are represented as Mean $\pm$ SEM of three replicates. One-way ANOVA

was used for the comparison between the groups and p values with 0.05 or less than 0.05 was considered significant. GraphPad Software (Avenida de la Playa, CA, USA) was used for all the analysis.

3.4 Results

3.4.1 TLR10 gene sequence alignment and protein expression in lung

Commercial TLR10 antibodies are not available specifically for cattle, dog, pig, and chicken. Therefore, we compared the sequence of human TLR10 peptide used to raise a commercial antibody with the TLR10 sequence of cattle, pig, dog and rat. According to the manufacturer, the anti-human TLR10 antibody was raised against a synthetic peptide of 32 amino acids corresponding from 80-111 of human TLR10 (Fig.3. 1A). The FASTA sequence of human TLR10 (GenBank Accession No. NM_030956) retrieved from NCBI was aligned against cattle (GenBank Accession No. NM_001076918), domestic pig (GenBank Accession No. NM_001030534), dog (GenBank Accession No. NM_001173127) and rat (GenBank Accession No. NM_001146035), using Clustal-W (EMBL-EBI, Cambridge, UK). The results indicated that the human TLR10 peptide used to generate the antibody had a homology of 71% with rat, 82% with dog, and 80% with both cattle and pig TLR10 proteins.

3.4.2 Western blots for TLR10

Western blotting of total lung protein extracts showed a band in 95 kDa region, indicating the expression of TLR10 protein in cattle, dog, and pig (Fig. 3.1B). The densitometric analysis (Fig. 3.1C) showed increased TLR10 expression in *M. haemolytica* infected cattle but not in influenza-infected pigs compared to their respective

controls. To analyze the changes in TLR10 expression during combined bacterial and viral infection, we performed the western blots with total lung protein isolated from chickens infected with *E. coli* and foal adenovirus (Figure 3.2A). We used densitometry to compare the expression of TLR10 between the normal and inflamed lungs. TLR10 expression was found significantly ($P<0.05$) increased in lungs from all infected chickens compared to the controls (Fig. 3.2B).

3.4.3 Expression and localization of TLR10 in normal and inflamed lungs

Lung sections from cattle (Fig. 3.3A), rat and dog (data not shown) stained with isotype-matched antibody showed no staining. The specificity of TLR10 antibody was further established by lack of staining in sections that were incubated with blocking peptide-antibody mixture (Fig. 3.3B). The lung sections reacted with von Willebrand Factor (vWF) (Fig. 3.3C), a vascular endothelial antigen, delineated the vascular endothelium.

TLR10 staining of control calf lung indicated the expression of the receptor in sub-epithelial area of bronchioles and sub-endothelial area of blood vessels (Fig. 3.3D). We observed TLR10 positive staining in alveolar septa as well. The TLR10 staining was much reduced in the *M. haemolytica* infected animals (Fig. 3.3E) compared to the controls.

TLR10 staining in a control pig lung was observed in sub-epithelial areas of bronchioles (Fig. 3.4A). The TLR10 staining is apparently increased in lungs from pigs infected with swine influenza virus (Fig. 3.4B) and increased positive staining noted in bronchial cartilage (asterisks in Fig. 3.4B). Alveolar septa in lungs from control pigs (Fig. 3.4C) showed negligible staining compared to those in lungs of infected pigs (Fig. 3.4D).

TLR10 staining in a control rat lung is seen in bronchioles but nearly absent in alveolar septa (Fig. 3.5A). Lung section from a rat treated with *E. coli* LPS shows much increased staining for TLR 10 in apical parts of bronchial epithelial cells, sub-epithelial areas (Fig. 3.5B), and alveolar septa (Fig. 3.5C).

Three sections from chicken lung show staining for von Willebrand Factor (Fig. 3.6A), alpha-smooth muscle actin (Fig. 3.6B) and TLR10 (Fig. 3.6C) in a blood vessel. They show presence of TLR10 in perivascular space while faint staining is noticed on the endothelial surface. In contrast, vWF and alpha-smooth muscle actin mainly reacted with endothelium and vascular smooth muscle, respectively.

We determined the fine localization of TLR10 in calf and pig lungs using immuno-electron microscopy. An electron micrograph of cattle lung shows TLR10 staining in alveolar epithelium, vascular endothelium, and cytoplasm, and nucleus of a pulmonary intravascular macrophage (Fig. 3.7A). Alveolar macrophages (Fig. 3.7B) also showed TLR10 staining in plasma membrane, nucleus and cytoplasm. TLR10 is also localized on the luminal surface and cytoplasm of lung capillary endothelial cells (Fig. 3.7C).

3.4.4 TLR10 expression in bovine neutrophils

We used isolated bovine neutrophils to further clarify the expression of TLR10 in these cells. Confocal microscopy of bovine neutrophils showed TLR10 on the plasma of normal neutrophils (Fig. 3.8). The TLR10 expression was increased especially in the cytoplasm of neutrophils treated with LPS (Fig. 3.8).

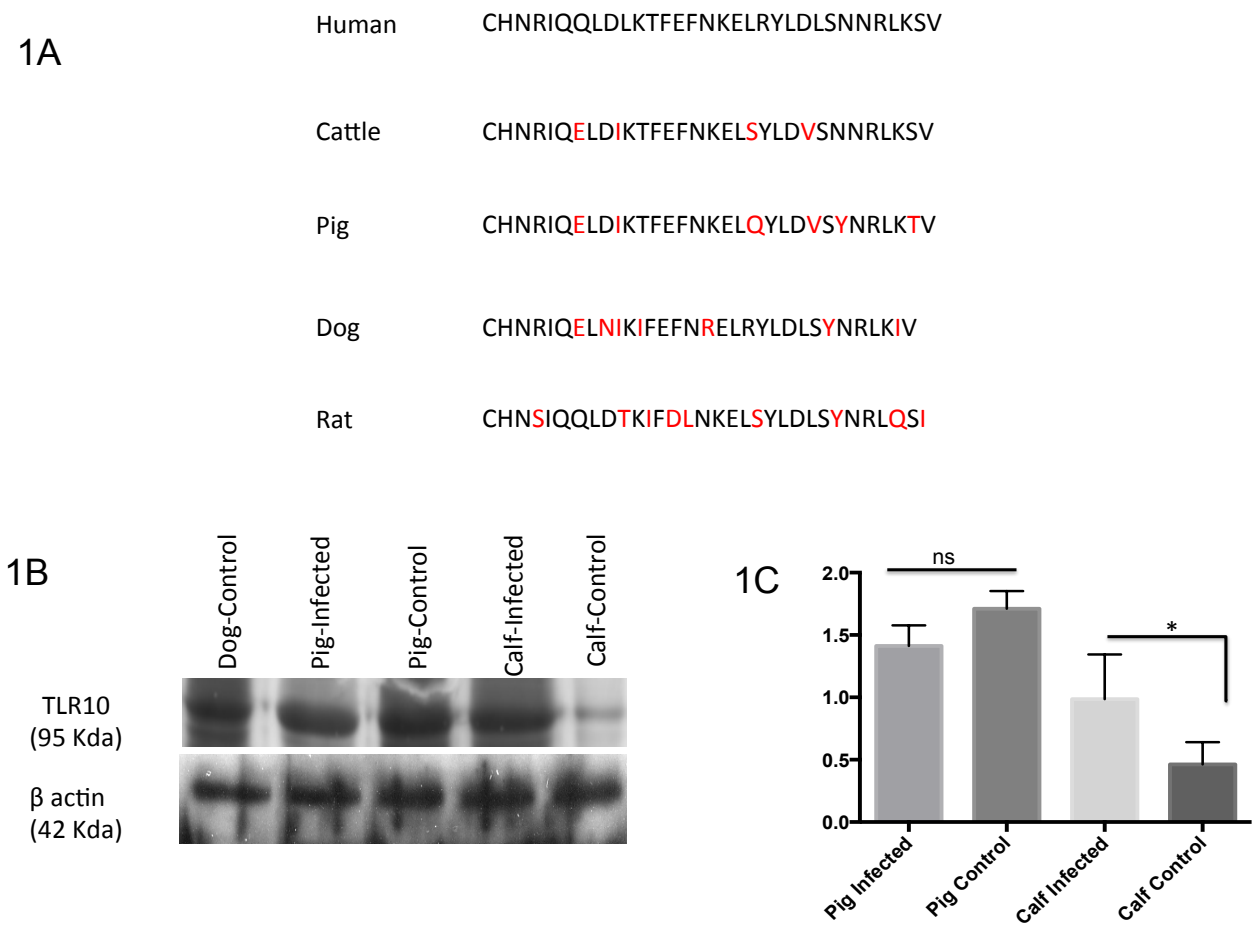


Figure 3. 1: TLR10 amino acid sequence comparison and TLR10 western blots

The comparison of human TLR10 amino acid sequence (Figure 1A) used for generating TLR10 antibody shows 80% homology with cattle and pig, 82% with dog, and 71% with rat corresponding to that detected by the antibody used. Figure 1B shows western blots from lung homogenates from cattle, pig, and dog. Each of the species shows a band of 95kD, which corresponds to the molecular weight of TLR10. The data showed significant increase in TLR10 expression lungs of calves infected with *M. haemolytica* compared to the controls (Figure 1C).

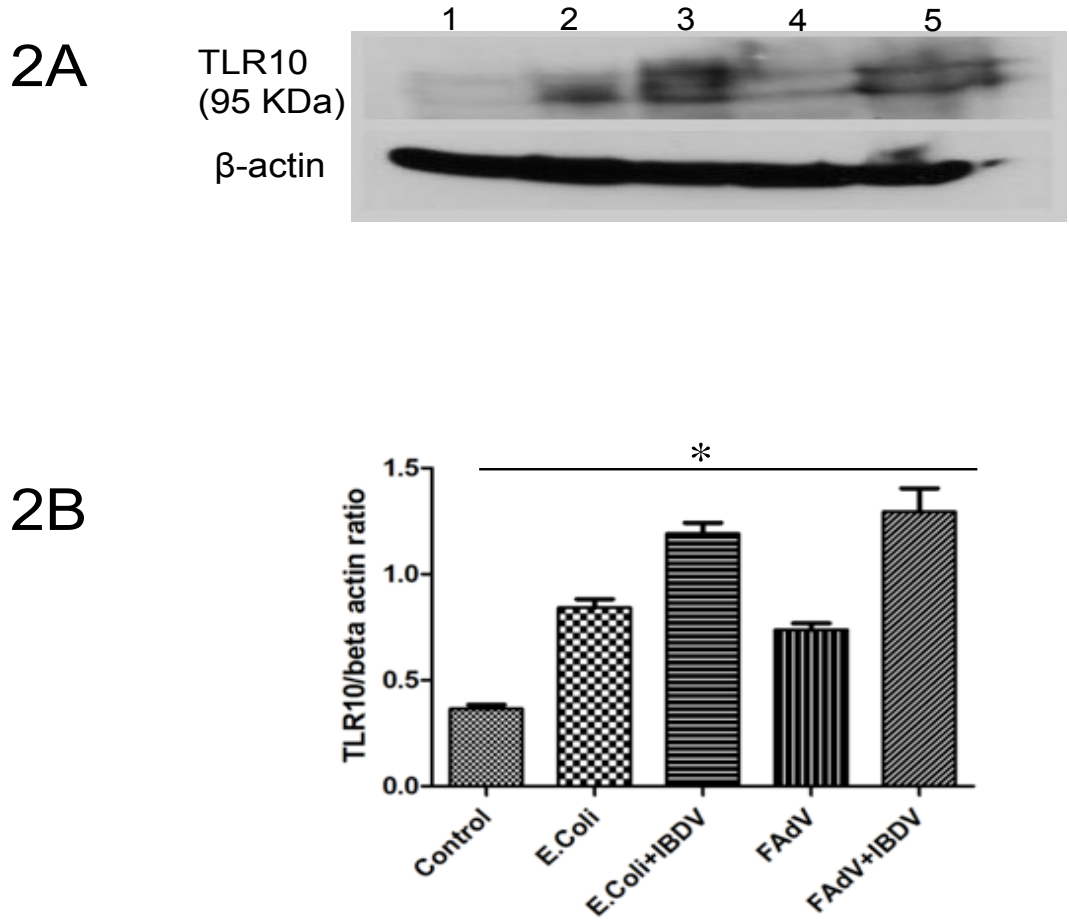


Figure 3. 2: Lung inflammation upregulated TLR10 expression

(Figure 2A) Immunoblot of lysates from control and infected chicken lungs show 95KDa protein bands corresponding to molecular weight of TLR10. Lane1: Control; Lane 2: *E. coli* infection; Lane 3: *E. coli* + IBDV infection; Lane 4: FAdV infection; Lane 5: FAdV + IBDV infection. Densitometric quantification (Figure 2B) showed significant increase in TLR10 expression in infected lungs compared to the controls (*=P<0.05).

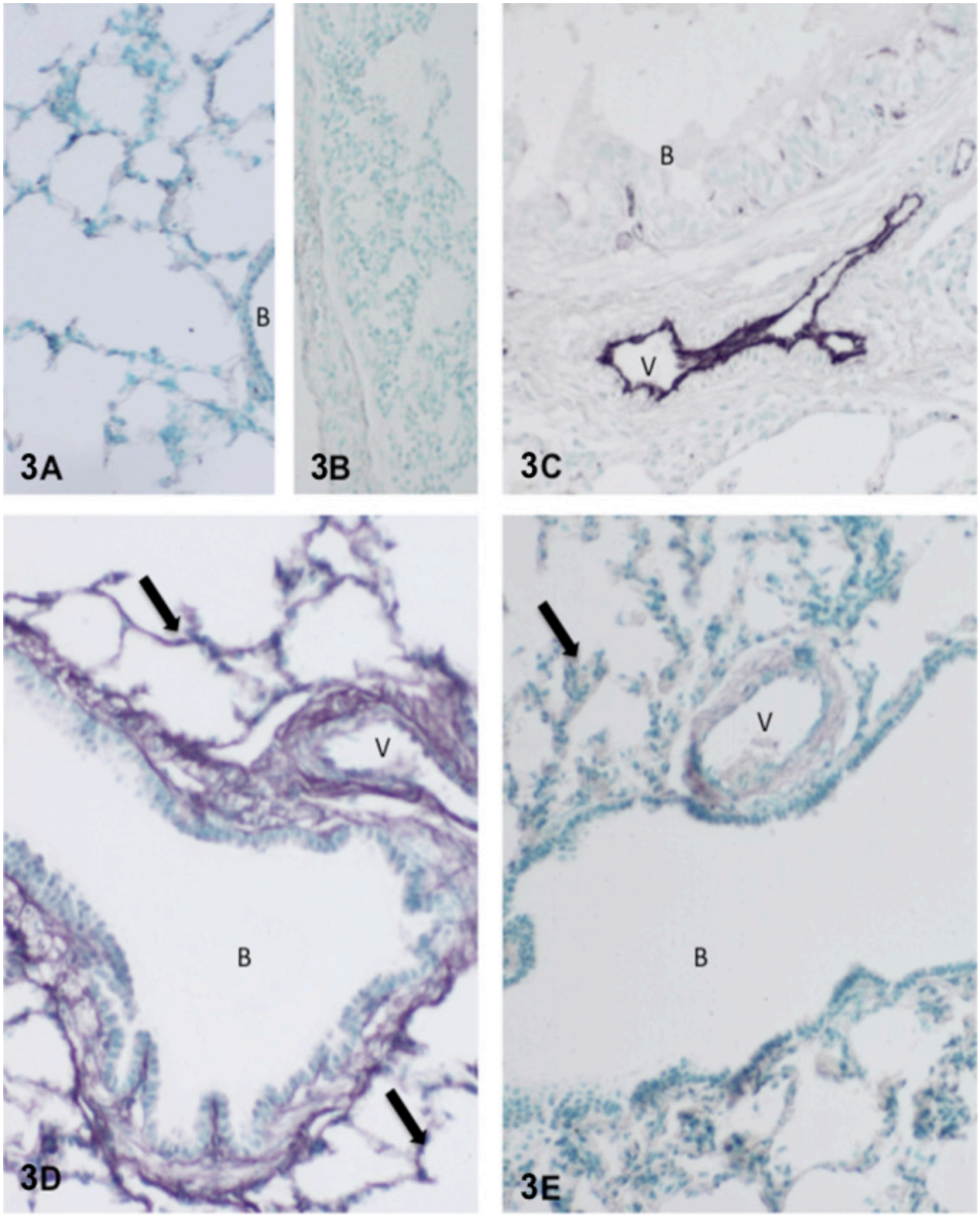
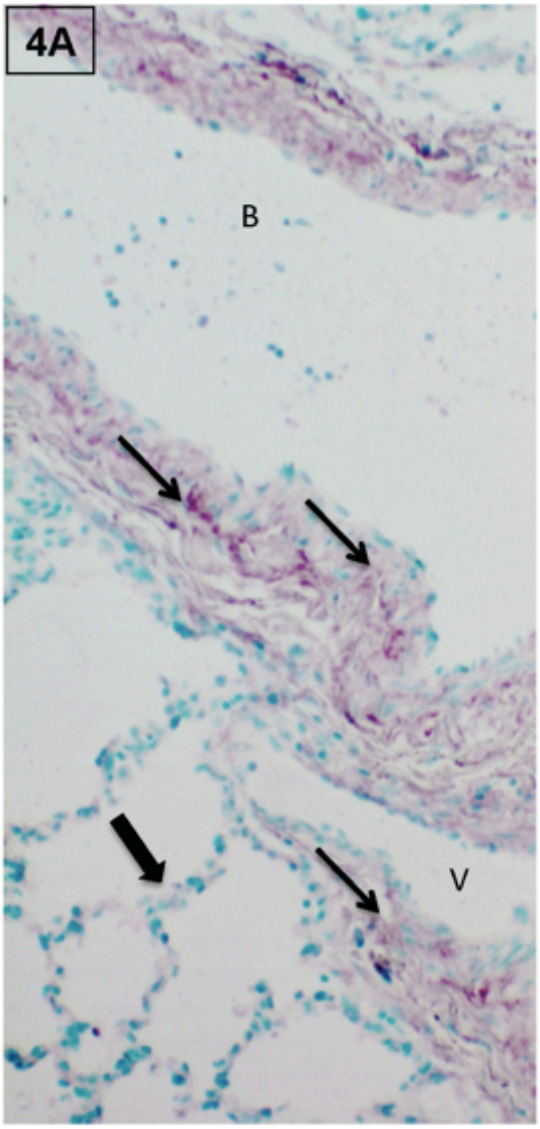
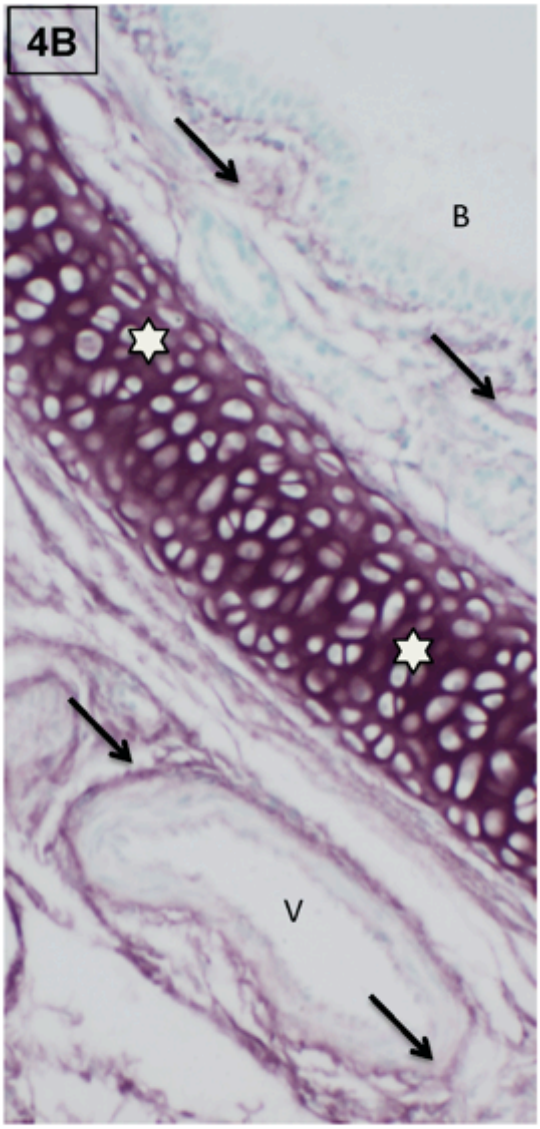
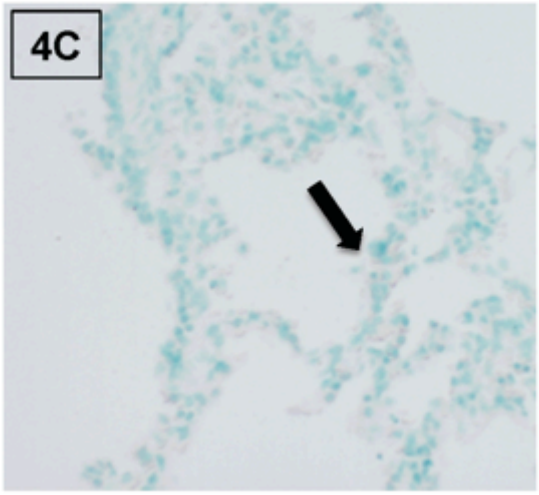

B
3A
3B
B
V
3C
V
B
3D
V
B
3E

Figure 3. 3: Immunohistochemistry of normal and inflamed calf lungs

Lung section from control cattle stained with isotype-matched antibody (Figure 3A) lacks staining. Figure 3B shows lack of staining a pig lung section where a blocking peptide was used in the protocol whereas the one stained with vWF antibody (Figure 3C) shows staining in endothelium (arrows) but not in bronchiolar epithelium. Lung section from a control animal (Figure 3D) shows staining (arrows) in sub-epithelial area of a bronchiole and sub-endothelial area of a blood vessel. Alveolar septa (thick arrows) also show TLR10 staining. The TLR10 staining is much reduced in a lung from a calf infected with *M. haemolytica* (Figure 3E) compared to the control (Figure 3D). 400X.


4A
B
V


4B
B
V


4C

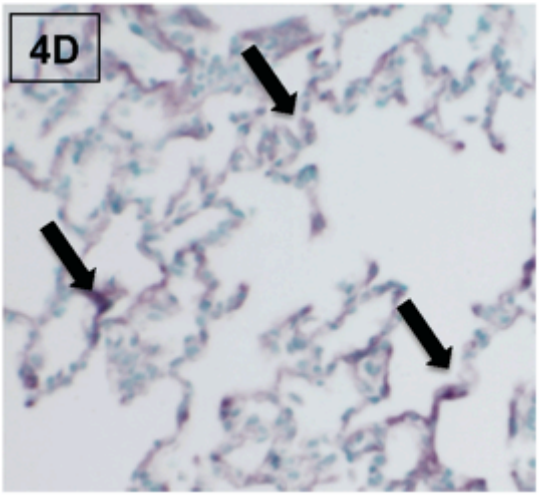

4D

Figure 3. 4: Immunohistochemistry of normal and inflamed pig lungs

Figure 4A shows TLR10 staining (arrows) in a control pig lung is observed in sub-epithelial areas of a bronchiole. The TLR10 staining (Figure 4B) is apparently increased in a lung from pig infected with influenza A. TLR10 positive staining was observed in the bronchial cartilage (shown in asterisks). TLR10 staining (Figure 4C) in the alveolar septa in control animals is negligible compared to those from the infected pigs (Figure 4D). 400X.

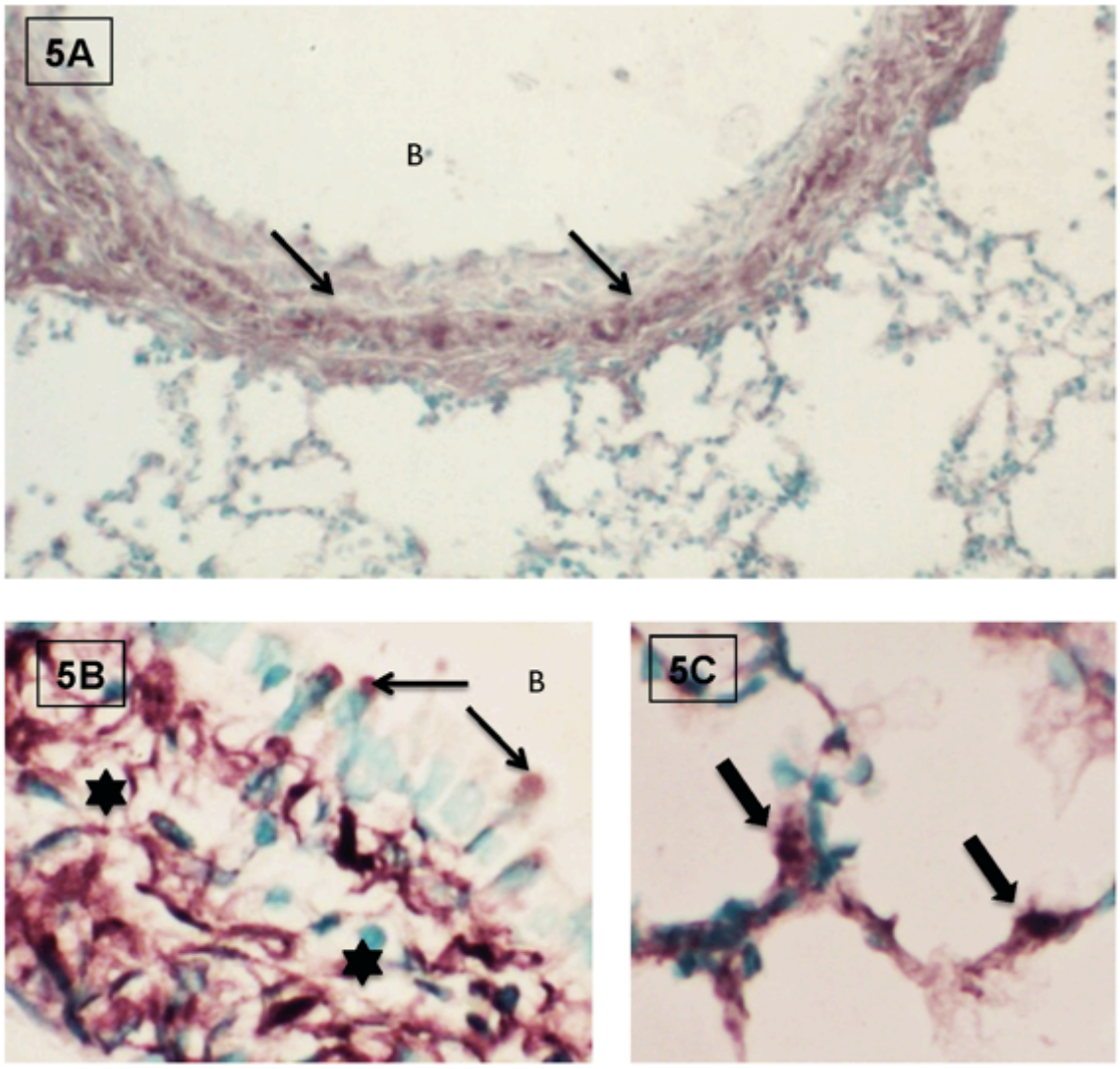
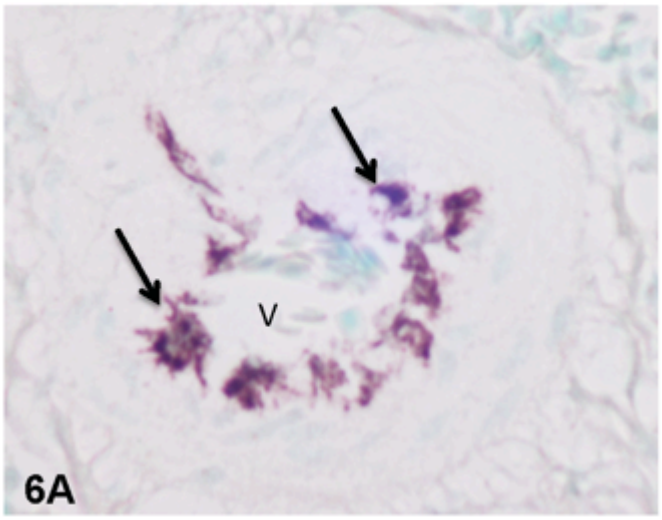
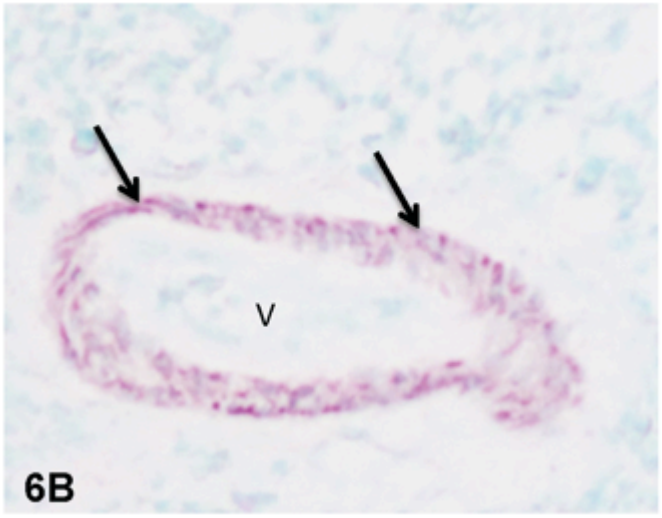


Figure 3. 5: Immunohistochemistry for rat lungs

TLR10 staining (Figure 5A) in a control rat lung is seen in a bronchiole but nearly absent in alveolar septa. Section from a lung from a rat treated with *E. coli* LPS shows staining in bronchial epithelial cells (arrows), sub-epithelial areas (asterisks) in figure 5B and alveolar septa in figure 5C. 400X (A) and 1000X (B&C).


V
6A


V
6B

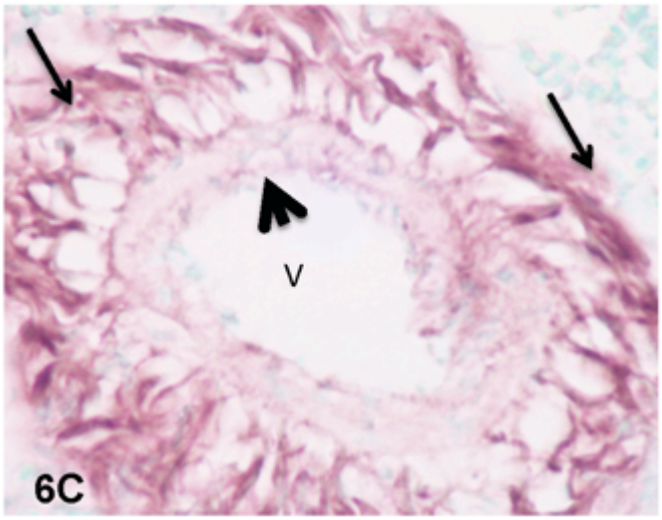
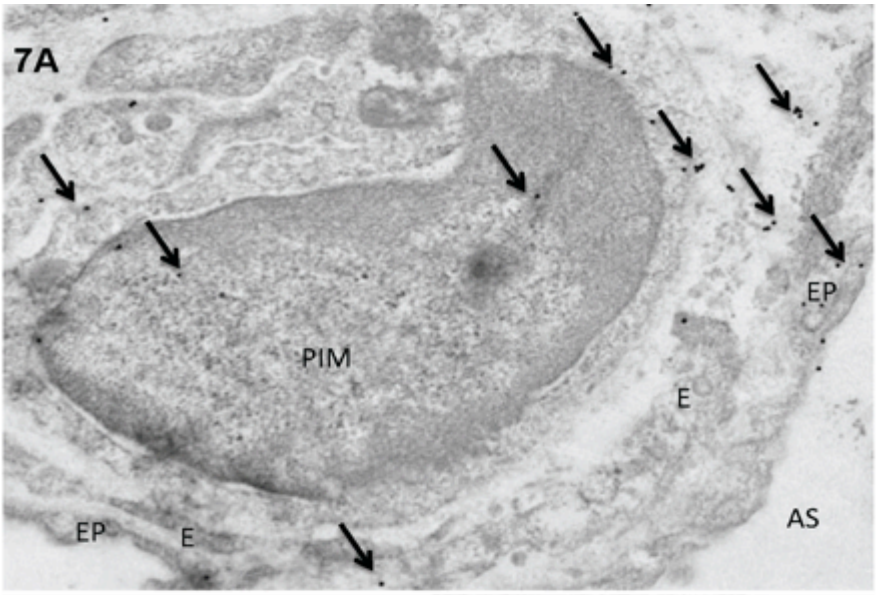
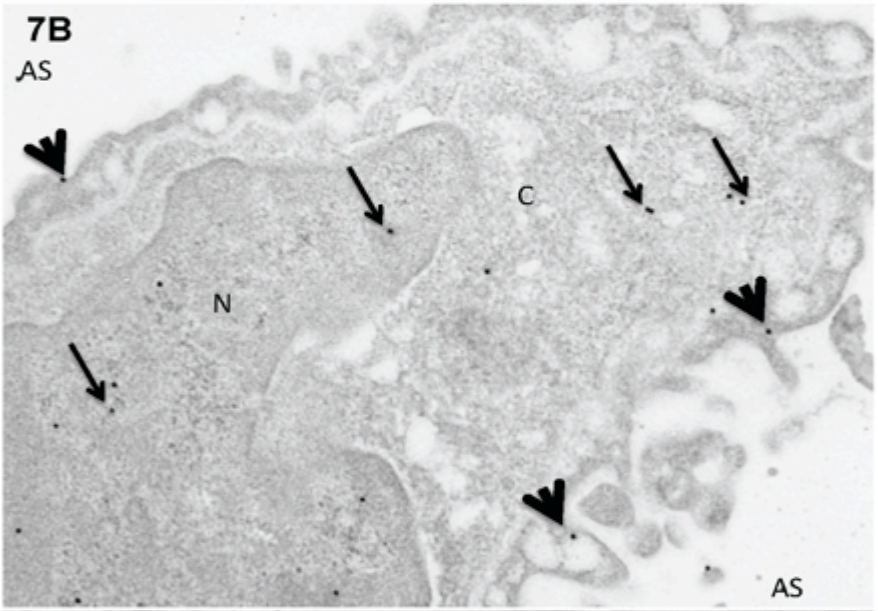

V
6C

Figure 3. 6: TLR10 staining in perivascular area of blood vessel

Three sections from chicken lung show staining (arrows) for von Willebrand Factor (Figure 6A), alpha-smooth muscle actin (Figure 6B) and TLR10 (Figure 6C) in a blood vessel (V). The sections show TLR10 mainly in perivascular space while faint staining is noticed on the endothelial surface while vWF and alpha-smooth muscle actin stain endothelium and smooth muscles, respectively. 400X


7A
PIM
EP
E
EP
E
AS


7B
AS
C
N
AS

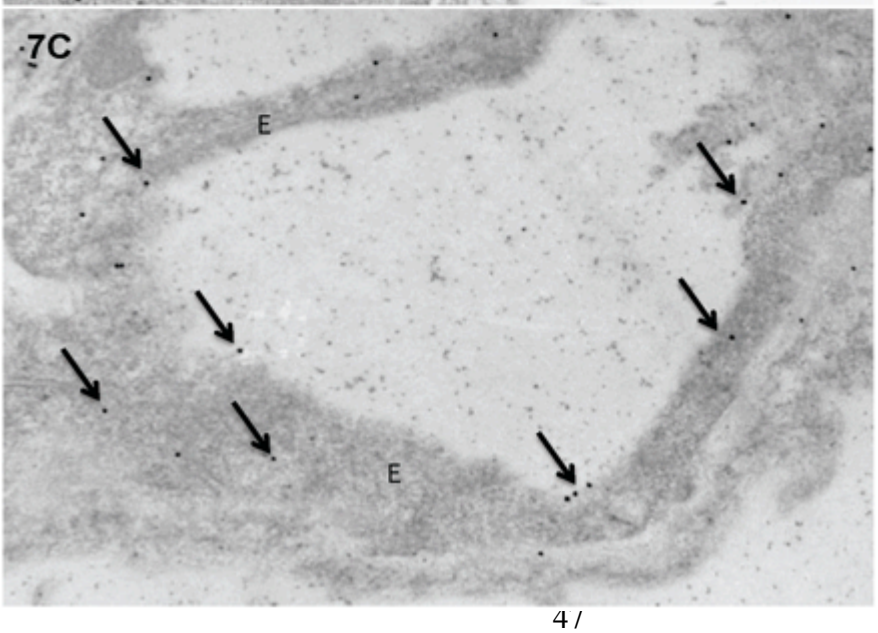

7C
E
E

Figure 3. 7: TLR10 immunogold electron microscopy in cattle lung

TLR10 staining (Figure 7A) (arrows) observed in a pulmonary intravascular macrophage (PIM), alveolar endothelium (EP) and endothelium (E). Figure 7B shows TLR10 staining (arrows) nucleus (N) and cytoplasm (C). The labeling is also observed on the plasma membrane (short arrows) in an alveolar macrophage in cattle lung. Figure 7C depicts TLR10 shows labeling (arrows) on luminal surface and cytoplasm of capillary endothelium (E). 13,000X

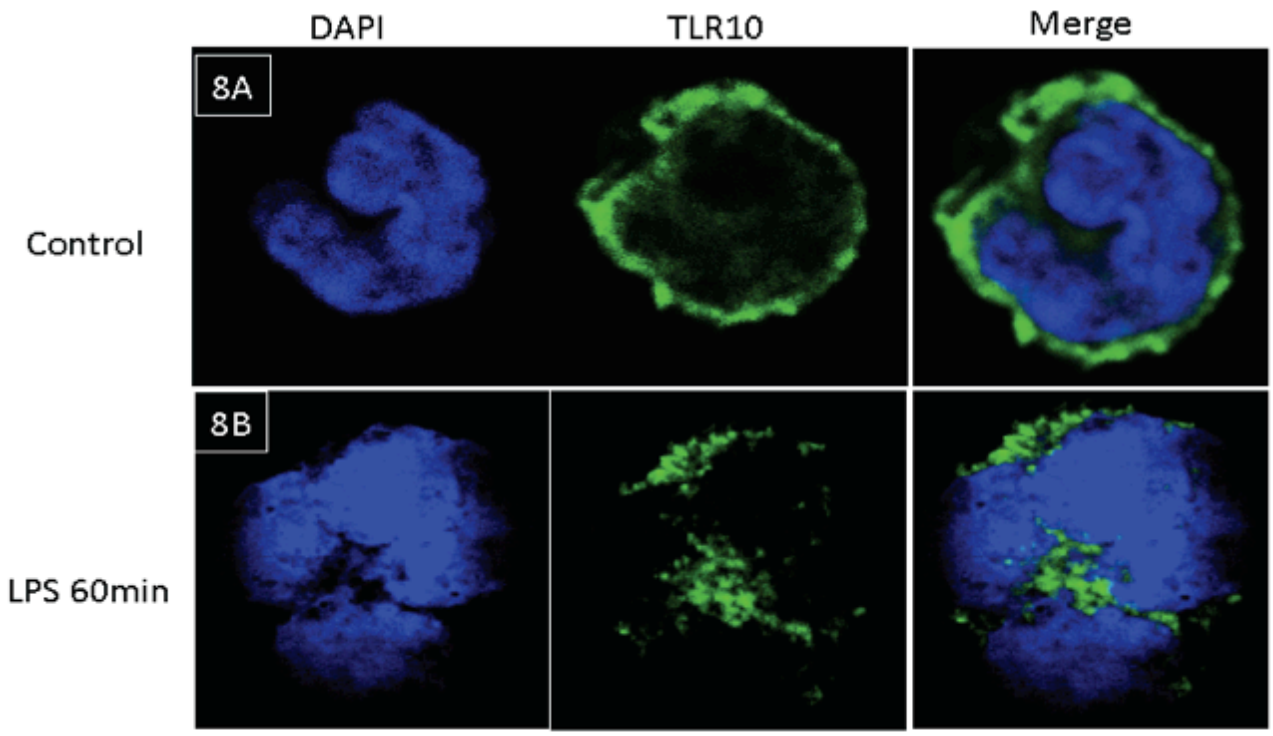


Figure 3. 8: TLR10 protein expression in bovine neutrophils

Control neutrophils (Figure 8A) show TLR10 staining (figure 8A). Neutrophils treated with *E. coli* LPS (8B) show altered expression of TLR10. 630X

3.5 Discussion

We report the first data on the expression of TLR10 in normal and inflamed lungs of cattle, pig, rat and chicken, and normal lungs of dog. In addition, we also show TLR10 expression in control and LPS-treated isolated bovine neutrophils. The work contributes standardization of a commercially available TLR10 antibody for use in multiple veterinary species. In addition to the use of antibody to detect TLR10 in lungs of various species reported in this paper, the antibody also reacted with TLR10 in liver of rats and skeletal muscle of calves (data not shown). While immuno-histochemistry and western blots showed changes in TLR10 expression in control and inflamed lungs, the immuno-electron microscopy provided data on subcellular localization of TLR10. Although the identity of a ligand for TLR10 is still elusive, the constitutive and altered expression of TLR10 in lungs may suggest its potential role in lung immunity.

Bacterial and viral infections, and associated economic losses are the major concerns in animal industry (Aschenbroich et al., 2013; Pomorska-Mol et al., 2013). Since TLRs have central roles in detection and initiation of inflammation (Bafica et al., 2005; Beutler, 2009), it is important to locate the tissue-cell specific expression of these receptors and any alterations in their expression in infected host. While many expression data are available for TLRs such as TLR4, TLR2 and TLR9, relatively little data have been published on TLR10 so far. Furthermore, most of the TLR10 expression data have been obtained in cultured cells or through the use of recombinant expression proteins (Guan et al., 2010; Kim et al., 2010; Selvarajoo et al., 2010). The challenge is further compounded in veterinary medical species where specific immunological reagents may not be available. Therefore, we characterized the commercially available anti-human

TLR10 antibody (Abcam; ab53631) to detect the 95-kDa-protein band in western blotting, corresponding to TLR10 protein. The peptide alignment of TLR10 protein from the multiple species showed the amino acid sequence similarity of 70%-90% with that of the peptide used to raise the antibody. We used this antibody for evaluating TLR10 expression with light and electron microscopic immunocytochemistry and western blots.

The western blots demonstrated TLR10 in lungs from all the species examined in this study. Furthermore, chicken lungs infected with *E. coli* and FAdV had increased expression of TLR10. The *in situ* localization of TLR10 was in the sub-epithelial areas in the airways, sub-endothelial areas of blood vessels, and the alveolar septa of the lungs. It appeared that TLR10 was mainly in the smooth muscles with little expression in the epithelial and endothelial cells. This was confirmed with staining of serial sections of lungs of chicken. It is interesting to note that while the light microscopic immunochemistry showed some basal TLR10 expression in lung septa, the fine localization with electron microscopic immunochemistry clearly defined the apical and cytoplasmic location of TLR10 in the septal cells. The presence of innate immune receptors in vascular endothelium provides an opportunity for the detection of blood-borne pathogens and their products (Andonegui et al., 2003). TLRs present in the endothelium activate the immune system upon an encounter with specific antigen (Sabroe et al., 2008). Bacteria such as *M. haemolytica* enter host body primarily through airborne route than directly in to the blood (Confer, 1993; Ewers et al., 2004). This leads to a critical role for the immune receptors in lung in activation of innate immune system to produce antibacterial or antiviral agents to clear the infection. The implications of reduced expression and roles of TLR10 in lungs of *M. haemolytica* infected cattle needs

to be confirmed and analyzed through further experiments. It is possible that the bacteria may directly suppress TLR10 in lung to gain advantage and to establish infection. The expression of TLR10 in epithelial and endothelial surfaces of the lung may be important in lung pathophysiology; however, the identification of TLR10 ligand is urgently needed to understand the physiological importance of epithelial and endothelial TLR10 in the lung.

Macrophages are major regulators of immune responses in the lung. Alveolar macrophages present in the alveoli detect and remove inhaled bacteria and dust particles. PIMs are present in species such as cattle, sheep, goat, pigs, and horse and phagocytose blood-borne particles (Winkler, 1988; Chitko-McKown et al., 1991; Singh, 2004; Schneberger et al., 2012). Our data show TLR10 in both alveolar and intravascular macrophages in lungs of cattle and pig. Interestingly, electron microscopic data showed TLR10 in the nuclei of macrophages. Previously, we have demonstrated similar expression of TLR4 and TLR9 in lung macrophages of cattle and horses (Singh Suri et al., 2006; Schneberger et al., 2009). These TLR10 data make it clear that alveolar macrophages and PIMs have the TLR machinery that may allow them to respond to a variety of microbial molecules.

Macrophages orchestrate immune responses through production of inflammatory molecules that recruit neutrophils. Neutrophils as first responders migrate towards the site of inflammation using chemoattractants and adhesion molecules, where they inactivate or destroy microbial products by producing proteolytic enzymes, antimicrobial proteins, and reactive oxygen species (Janeway and Medzhitov, 2002; Brinkmann et al., 2004). Because of the importance of neutrophils in acute inflammation, we examined TLR10

expression in them. Our data show that neutrophils themselves express TLR10, and *in vitro* activation with LPS changed the localization of TLR10 in neutrophils. Therefore, neutrophil TLR10 may also have the capability to detect yet to be identified TLR10 ligand.

Taken together these are the first data on the expression of TLR10 in normal and inflamed lungs of multiple veterinary species as well as bovine neutrophils *in vitro*. Once the identity of the ligand for TLR10 is revealed, these data will be foundational to examine their roles in lung immunity.

CHAPTER 4: TOLL-LIKE RECEPTOR 10 EXPRESSION, LOCALIZATION AND ROLE IN CHEMOTAXIS IN HUMAN NEUTROPHILS ON BACTERIAL LIPOPOLYSACCHARIDE CHALLENGE

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Running title: Expression and functional characterization of hTLR10 in neutrophils

Author contributions: Y.B, and B.S conceived the idea and Y.B performed research. S.C helped to perform IEM. Y.B and B.S wrote the manuscript.

Keywords: TLR10, TLR4, lipopolysaccharide, neutrophils, flotallin-1, ROS, NF-κB, chemotaxis

4.1 Abstract

Toll-like receptors are innate immune receptors that play critical role in pathogen associated molecular pattern recognition. TLR10 is recently identified and its signaling pathway is still unclear. To study the expression pattern of TLR10 in primary immune cells, we examined TLR10 protein expression in normal and *E. coli* lipopolysaccharide (LPS) activated human neutrophils. Human neutrophils challenged with LPS showed a decreased TLR10 expression at 90min. Flow cytometry confirmed the reduced surface expression of TLR10 in 90min during LPS treatment. Confocal microscopy showed cytosolic and nuclear distribution of TLR10 in normal and activated neutrophils. TLR10 in LPS-activated neutrophils colocalized with flotallin-1, a lipid raft marker, and EEA-1, an early endosomal marker, to suggest its endocytosis. Colocalization analysis with TLR4 showed increased colocalization during 60min and a gradual decrease during the time. Decreased cytoplasmic localization of TLR10 was observed in TLR4 neutralized neutrophils when stimulated with LPS. Reactive Oxygen Species (ROS) depletion and neutralization of p65 in LPS-treated neutrophils resulted in decreased TLR10 expression. Live cell imaging of LPS-activated neutrophils showed TLR10 translocation in the leading edge and the role of TLR10 in chemotaxis was verified by the inability of TLR10 knockdown neutrophils to move towards fMLP. However, TLR10 knockdown did not change the expression of key proteins of actin nucleation process, ARP-3 and Diap1. Together, our findings show that LPS alters TLR10 expression, and TLR10 plays role in neutrophil chemotaxis.

4.2 Introduction

Toll-like Receptors (TLRs) are the innate immune receptors and their name comes from Toll receptor, which was identified for its roles in embryonic development in Drosophila (Hashimoto et al., 1988). TLRs recognize ligands derived from various microorganisms including bacteria, viruses, protozoa, and fungi (Alexopoulou et al., 2001; Hayashi et al., 2001; Miettinen et al., 2001; Takeuchi and Akira, 2002; Lund et al., 2003; Diebold et al., 2004; Heil et al., 2004; Latz et al., 2004; Takeda and Akira, 2004; Akira et al., 2006). So far, ten TLRs (1-10) have been reported in humans and 13 TLRs (1-9 and 11-13) in mouse. TLR10 is recently identified and is non-functional in mice due to retroviral insertions (Selvarajoo et al., 2010). Co-immunoprecipitation studies show that activated TLR10 forms homodimers, and makes heterodimers with TLR1/2 via extra cellular domains (Hasan et al., 2005). The structural studies of the cytoplasmic domains of TLR10 revealed MyD88 as the probable adaptor molecule (Nyman et al., 2008). Currently, the information about TLR10 signaling pathway, ligand identity, and function is scarce. Hence, it remains as an "orphan receptor" of the innate immune system.

TLR10 protein is expressed in organs such as lymph nodes, spleen, thymus, lungs, and immune cells such as macrophages and neutrophils (Chuang and Ulevitch, 2001). We have found that TLR10 is widely expressed in lungs of species such as pig, dog, cattle, and chicken and that its expression is altered in inflamed lungs (Balachandran et al., 2015). The expression of TLR10 mRNA is found in early B cell development and the translation commitment mainly observed during B cell differentiation (Bourke et al., 2003). Recent studies indicate the role of TLR10 in Crohn's disease, an inflammatory

bowel disease characterized by mucosal dysfunction (Abad et al., 2011). TLR10 is identified as the key receptor in the innate immune response against *L. monocytogenes* through upregulation of chemokines CCL-20, CCL-1 and IL-8 through the nuclear translocation of NF-κB (Regan et al., 2013). Kim and colleagues showed the TLR10 activation in THP-1 cell line under hypoxic conditions and proposed intracellular reactive oxygen species (ROS) and NF-κB activation as downstream effects of the activation (Kim et al., 2010). But recently, Oosting and colleagues reported that the TLR10 is an anti-inflammatory receptor because its neutralization in peripheral blood mononuclear cells increased production of proinflammatory cytokines such as IL-1β (Oosting et al., 2014). Polymorphisms of TLR1- TLR6- TLR10 super family have been reported in diseases such as sarcoidosis and prostate cancer (Chen et al., 2007; Veltkamp et al., 2012). Taken together, although the few available data implicate TLR10 in inflammatory response, the regulation of TLR10 expression and immune functions remains poorly understood.

Neutrophils are central to the genesis of acute inflammation generated in response to bacteria or their products such as LPS (Standish and Weiser, 2009; Kumar and Sharma, 2010). LPS activates neutrophils upon binding to TLR4 expressed on their surface. Activated neutrophils migrate into sites of inflammation, produce anti-microbial products, undergo NETosis and kill bacteria (Branzk and Papayannopoulos, 2013). The anti-microbial products such as ROS produced by activated neutrophils also cause significant damage to the tissues, which is credited with morbidity and mortality (Lee et al., 2003). Neutrophils must extravasate from blood vessels to reach extravascular site of infection to tackle microbes (Mathias et al., 2006). The neutrophils move through a

complex series of cellular and molecular events regulated by adhesive proteins, chemo-attractants, and cytoskeletal reorganization. Kubes and colleagues have previously demonstrated the role of TLR4 in neutrophil chemotaxis in the lung (Andonegui et al., 2003). However, there are no data on the regulation of TLR10 expression and its role in neutrophil chemotaxis.

To gain an understanding of the regulation of TLR10 expression and to address the issue of its role in neutrophil chemotaxis, we performed a series of *in vitro* studies. The data show that TLR10 is expressed in human neutrophils and LPS treatment altered TLR10 expression in human neutrophils. TLR10 gene knockdown induced chemotactic arrest in HL-60 derived neutrophils but it did not affect the expression of actin nucleation key proteins.

4.3 Materials and Methods

4.3.1 Reagents

E. coli lipopolysaccharide (L6529) was obtained from Sigma Chemicals (St. Louis, USA). Anti-TLR10 polyclonal, anti-beta actin, anti-EEA1, anti-mouse IgG- FITC and anti-mouse IgG_1-FITC were from Abcam (Cambridge, MA, USA), and anti-flotillin-1 from Santa Cruz Biotechnology (California, USA). TLR4 neutralizing antibody and anti-Gr1 antibody commercially purchased from R&D systems (Minneapolis, USA). Peroxidase Substrate kit from Vector Laboratory Inc., (Burlingame, USA) and protein assay kit obtained Bio-Rad (Mississauga, Canada). RPMI-1640 purchased from ATCC (Manassas, USA), Reactive oxygen species detection kit was obtained from Invitrogen (Burlington, Canada) and all other chemicals from Sigma (St. Louis, MO, USA).

4.3.2 Ethical approval for human research

Study on human neutrophils from healthy volunteers, was approved by Research Ethics Board of University of Saskatchewan, Saskatoon Canada. Licensed and trained personnel collected blood and got consent from all the volunteers.

4.3.3 Isolation of neutrophils from human blood and stimulation

Blood was collected intravenously from healthy volunteers and carefully added to the density gradient of Histopaque- 1119, 1083 and 1077 as described previously (Oh et al., 2008). Following centrifugation at 700*g* for 20min at room temperature, the layer between Histopaque 1119 and 1083 was aspirated and red blood cells were removed through hypotonic lysis. The cell pellet was suspended in RPMI 1640 with 10% FBS and viability checked by using tryphan blue. Cells were stimulated with bacterial LPS (1µg/ml) and harvested at 60min, 90min and 120min time interval.

4.3.4 Flow Cytometry for TLR10 surface expression in neutrophils

FACSCalibur flow cytometer (BD Biosciences, Mississauga, Canada) was used to perform the flow cytometry assays. LPS treated cells were treated with Fc receptor blocker in order to reduce the background staining followed by the incubation with mouse antihuman TLR10 monoclonal antibody (0.35µg/ test; ab113446, Abcam) for 30 min at 4°C. PE conjugated Gr-1 (0.30µg/ test; 108407, Biolegend) used as marker for preliminary experiments to determine the neutrophil population and FITC conjugated

anti-mouse IgG_1as isotype control. FITC labeled anti-mouse IgG antibody used against TLR10.

4.3.5 Confocal microscopy for TLR10, EEA1, Flotilin-1, TLR4, ROS and actin

Confocal microscopy was performed using Leica TCS SP5 (Leica Microsystems, Germany) with 63X/1.2 oil immersion objective for image acquisition. Isolated neutrophils were incubated at 37°C and 5% CO_2 in RPMI-1640 supplemented with 10% FBS and stimulated with *E. coli* LPS for 60min, 90min and 120min. Resting cells without LPS challenge in the same conditions were used as control for the experiment. Cells were fixed in 4% paraformaldehyde and permeabilized using 0.01% Triton X-100. Blocking with 5% BSA followed by primary antibodies against TLR10 (1:250), early endosomal marker, EEA1 (1:200), Flotilin-1 (1:150), Alexa 488- phallodin (165nM) according to the experiment and incubated 1hr at room temperature (Daniel and Kai, 2010). Fluorescent-labeled secondary antibodies incubation was done for 30min at room temperature. DAPI was used to stain the nuclei. TLR4 neutralizing antibody (5µg/ml) was co-incubated with isolated neutrophils prior to LPS treatment and reactive oxygen species production was analyzed as marker for the receptor neutralization.

To evaluate TLR10 translocation in activated live neutrophils, isolated human neutrophils were stained with FITC conjugated TLR10 antibody and washed gently to remove all unbounded dyes. The cells were resuspended in 1X sterile PBS and 150µl of the suspension was added to the center of glass bottom culture dish (MatTek Corporation, 200 Homer Avenue, MA, USA) providing 37°C. Neutrophil activation was performed by

adding LPS (1μg/ml) to the cell suspension. Live cell activity was recorded for 120 min using time-lapse option.

4.3.6 Western blotting for TLR10, Diap1 and ARP3

After activation with LPS, isolated primary human neutrophils and HL-60 derived neutrophils (control and TLR10 knockdown) were lysed in RIPA buffer (Sigma, St. Louis, USA) with protease inhibitor cocktail. Immunoblots were prepared by anti-TLR10 antibody (1:600, 1mg/ml; Abcam, Cambridge, USA), anti-Diap1 antibody (1:500, Cell Signaling Tech. USA), anti-ARP3 antibody (1:400, Cell Signaling Tech. USA). Anti-β-actin (1:1000, Abcam, Cambridge, USA) was used as loading control. HRP-conjugated anti-goat or anti-rabbit (1:2000; DAKO, Burlington, Canada) was used as secondary antibody. Hybridization signals were detected in Amersham ECL western blotting detection reagents (GE Health care, Mississauga, Canada). Blots were digitally imaged and contrast adjustments were applied to all parts of the figure in an unbiased manner. Represented lanes indicate that only parts of the blot for better visualization. Quantification of bands from immunoblots was performed with densitometry in Adobe Photoshop CS6 (San Jose, USA).

4.3.7 Quantitative RT-PCR for TLR10

Total RNA was isolated from LPS-treated human neutrophils using Qiagen RNeasy Mini kit followed by the treatment with RNase free DNase (Qiagen, Ontario, Canada) according to the manufacturer's instructions. RNA was quantified by Nanodrop method and cDNA was prepared using Quantitect Reverse Transcription Kit (Qiagen,

Ontario, Canada). Quantitative real time PCR was performed using Stratagene MX3005P PCR instrument and brilliant SYBR Green QPCR kit (Agilent Technologies, Santa Clara, USA) was used for the reaction. TLR10 (FP: 3' ACTTTGCCCACCACAATCTC 5' and RP: 3' CCCAGAAAAGCCCACATTTA 5') and GAPDH (FP: 3' GAGTCAACGGATTTGGTCGT 5' and RP: 3' TTGATTTTGGAGGGATCTCG 5') primers were obtained from Invitrogen (Burlington, Canada). ROX was used as reference dye for the PCR reaction. Specificity of the reaction was measured with non-template and no-reverse transcriptase controls and analysis of melting curves. GAPDH was used for normalization of the expression.

4.3.8 Immunoelectron microscopy for TLR10 expression in neutrophils

Immunoelectron microscopy was performed on the neutrophils fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Nonspecific binding was blocked by incubating with 1% BSA and 0.1% Tween 20 in 1X TBS (pH 7.9) for 30min. The cells were incubated with primary goat anti-human polyclonal antibody (1:30) for 1hr at 25°C. Tissue sections were washed with TBS and incubated in 15mn gold particle labeled secondary antibody for 1hr (Singh Suri et al., 2006).

4.3.9 Small interference RNA induced TLR10 gene silencing in HL-60 cell lines

HL-60 cell line was transfected with commercially available siRNA transfection reagent using liposomes (Santa Cruz Biotechnology, 10410 Dallas, USA) after obtaining 80% confluence in culture as per manufacturer's instructions. In brief, 80nM of siRNA

for TLR10 (sc-40272; Santa Cruz Biotechnology) and missense oligonucleotide negative control (sc-36869; Santa Cruz Biotechnology) were incubated with transfection reagent for 45min to form transfection reagent-siRNA complex and added to the cells washed with transfection medium. HL-60 cells were incubated for 8hr in the transfection mixture and were recovered by washing with RPMI-1640. Transfected cells were incubated in complete RPMI-1640 medium for 48hr followed by their differentiation into neutrophil lineage through incubation in 1.3% DMSO for 5 days. Visual confirmation of differentiation was done by light microscopy.

4.3.10 Statistical analysis

One-way ANOVA was used for the comparison between the groups and *p* values with 0.05 or less than 0.05 was considered significant. Results are represented as Mean $\pm$ SEM of three replicates. Graph Pad Software (Avenida de la Playa, CA, USA) was used for all the analysis.

4.4 Results

4.4.1 Bacterial lipopolysaccharide alters the expression of TLR10 in human neutrophils

First, we examined the effect of bacterial LPS on the expression of TLR10 in human neutrophils. As shown in Figure 4.1A, the LPS treatment altered the localization of TLR10 in human neutrophils. The control neutrophils expressed TLR10 on their plasma membrane whereas at 60min of LPS treatment, TLR10 was observed mainly in the cytoplasmic vesicles. However, TLR10 staining in the cytoplasm was diffused at

90min of LPS treatment and reappeared on the membrane at 120min. We also noticed TLR10 expression in the nuclei of control as well as LPS-treated neutrophils. Flow cytometry confirmed reduction and reappearance of surface expression of TLR10 at 90min and 120min of LPS-treatment, respectively (Fig. 4.1B). We examined TLR10 expression in LPS-stimulated human neutrophils using western blotting (Fig. 4.1C, D). The TLR10 expression was reduced at 90min compared to control, 60min and 120min of the LPS treatment ($p<0.05$). Lastly, we determined transcriptional level expression of TLR10 with quantitative RT-PCR and found an increase in mRNA level of TLR10 at 60min of LPS-treatment compared to the controls (*Appendix: Supplementary information. 4.1*).

4.4.2 LPS induces the lipid raft mediated endocytosis of TLR10

We analyzed whether increase in cytoplasmic localization of TLR10 is through endocytosis. Quantitative colocalization of TLR10 with early endosomal antigen-1 (EEA-1), an endosomal marker protein, was performed at 60min, 90min, and 120min of the LPS treatment. Figure 4.2A shows TLR10-EEA1 colocalization in the cytoplasm of neutrophils at 60min and 90min treatment. At 120min of the LPS treatment, we observed colocalization signal on the plasma membrane compared to 60min and 90min of the treatment. The relocalization of TLR10 on plasma membrane at 120min was consistent with the observation in Figure 4.1A. Quantification of colocalization in terms of Pearson's Coefficient ($p<0.05$) is shown in Fig. 4.2B. The incubation of cells at $4^{o}C$ impaired increase in cytoplasmic localization of TLR10 to suggest receptor-mediated internalization of TLR10 (*Appendix: Supplementary information.4. 2*).

To determine the role of lipid rafts in TLR10 endocytosis, we examined the colocalization of TLR10 with flotillin-1, a resident lipid raft protein. The data show significant increase in colocalization of TLR10 with flotillin-1 after the neutrophil activation by LPS. We used methyl-beta-cyclodextrin (β-MCD; 1mM for 30min) to disrupt the lipid raft and this led to the inhibition of TLR10 endocytosis (*Appendix: Supplementary information.4. 3*). Figure 4.2C shows plasma membrane staining of flotillin-1 in control neutrophils. Fluorescent intensity of colocalization signal of TLR10 and flotillin-1 was increased at LPS 60min compared to the control neutrophils. Flotillin-1 staining on plasma membrane returned to control values at 120min of LPS treatment. Figure 4.2D represents the quantification of colocalization in terms of Pearson's Coefficient ($p<0.05$).

The ultra-cellular localization of TLR10 was confirmed with immunoelectron microscopy of control neutrophils (Fig. 4.3A). Neutrophils treated with LPS for 60min showed TLR10 aggregation on neutrophil pseudopods (Figure 4.3B). TLR10 was detected on plasma membrane, cytoplasm and nucleus of neutrophils at 120min of LPS treatment (Fig. 4.3C).

4.4.3 TLR10 colocalizes with TLR4 in LPS activated human neutrophils

Since bacterial lipopolysaccharide activates TLR4, we decided to examine the changes in TLR4 along with TLR10 expression on LPS challenge (Fig. 4.4A). At 60min of the LPS treatment, cytoplasmic colocalization of TLR10 with TLR4 was significantly increased compared to control neutrophils as well as those at 90min and 120min of LPS treatment ($p<0.05$; Fig. 4.4B).

4.4.4 Bacterial lipopolysaccharide-induced TLR4 activation regulates TLR10 expression and localization dynamics through reactive oxygen species

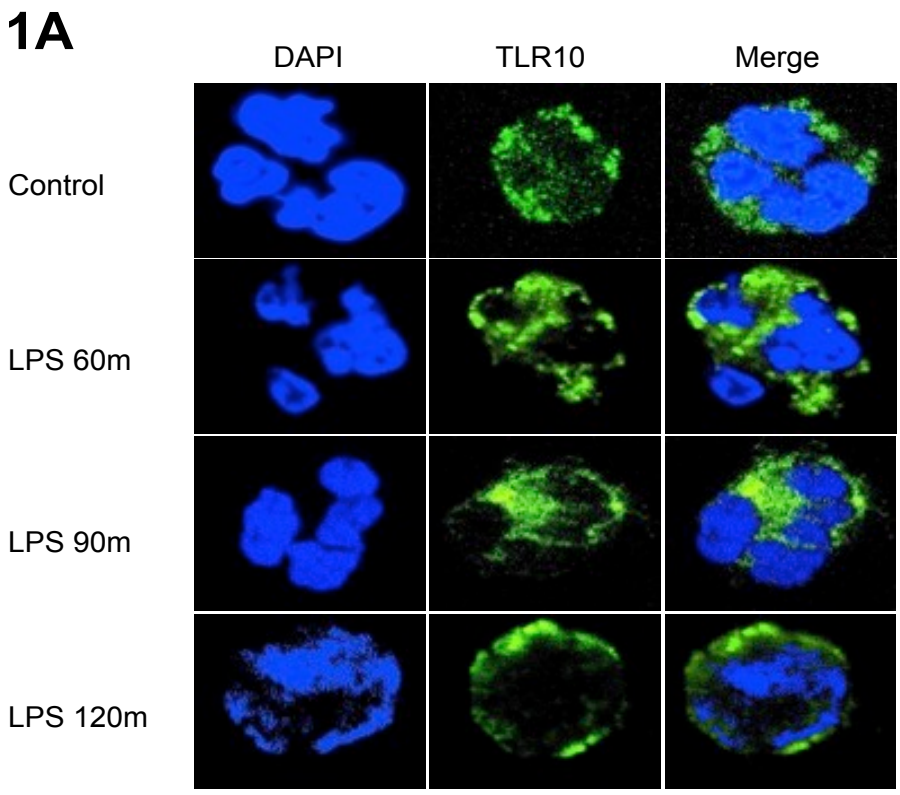
Bacterial LPS activates TLR4 that leads to the nuclear translocation of NF-κB, and production of reactive oxygen species (ROS) and proinflammatory cytokines (Yamada et al., 2006). Therefore, we assessed the role of LPS-induced TLR4 activation in TLR10 expression. TLR4 signaling pathway was inhibited with a TLR4 neutralizing antibody (5μg/ml) and production of ROS was monitored as marker for TLR4 neutralization. ROS production from TLR4-neutralized and LPS-treated neutrophils was significantly diminished when compared with LPS treated cells without neutralizing antibody (Fig. 4.5A). TLR10 expression was examined in untreated control cells, LPS-treated cells, and the cells treated with LPS for 60min and TLR4 neutralizing antibody. TLR4 neutralization in LPS-treated neutrophils significantly reduced TLR10 expression compared to the controls (Fig. 4.5B-C).

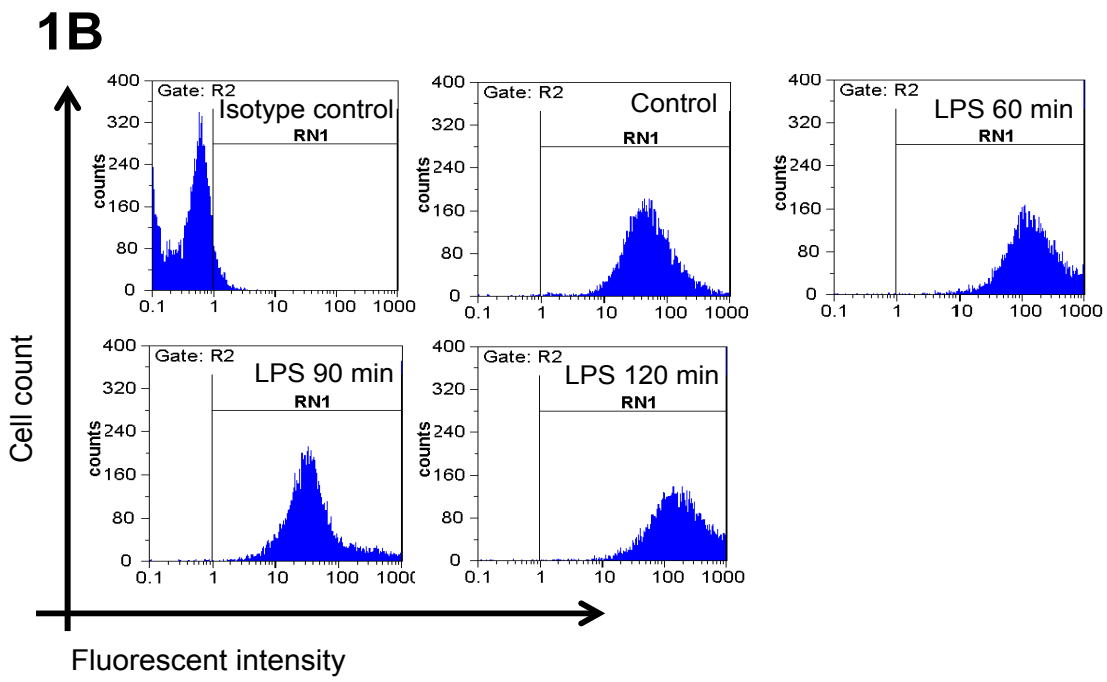
Since ROS production is characterized as one of the major effector events in LPS-treated neutrophils, we assessed the role of ROS in TLR10 expression. It has been demonstrated that LPS can rapidly induce ROS production through NADPH oxidase activation and lead to endothelial necrosis (Simon and Fernández, 2009). We used Trifluorocarbonylcyanide Phenylhydrazone (FCCP) as an uncoupler of oxidative phosphorylation and electron transport chain to prevent ROS induction in LPS treated neutrophils. Neutrophils treated with FCCP (5μg/ml) prior to incubation with LPS for 60min did not show an increase in TLR10 expression and cytoplasmic localization when compared with LPS-treated as well as untreated controls (Fig. 4.6A). Furthermore, we

analyzed the effect of nuclear translocation of p65 on TLR10 expression. Neutralization of p65 using an inhibitor peptide also reduced TLR10 expression (Fig. 4.6B). Taken together, these data show that ROS production and nuclear translocation of NF-κB after TLR4 activation by LPS regulates TLR10 expression.

4.4.5 TLR10 knockdown inhibits neutrophil chemotaxis

To examine the dynamic localization of TLR10 in live LPS-activated human neutrophils, we performed live single cell imaging. The data show FITC-labeled TLR10 preferentially localized to the leading edge of the neutrophils (Fig. 4.7A*, Supplementary information. 5- video.1*). Because human neutrophils are short-lived cells in culture, we used HL-60 cell line to assess the role of TLR10 in neutrophil chemotaxis. The effective knockdown of TLR10 in HL-60 cells (Fig. 4.7B-C) led to significant reduction in their chemotaxis towards fMLP (50nM) (Fig. 4.7D). To understand the underlying mechanism of regulation of chemotaxis by TLR10, we analyzed the changes in the expression of ARP3 and Diap1, which are key proteins involved in the process of actin nucleation. The Western blot data did not show differences in the expression of ARP3 and Diap1 (Fig. 4.8). Confocal imaging on control and TLR10 knockdown neutrophils confirmed the inability of TLR10 knockdown neutrophils to form the pseudopodia against fMLP stimulus (Fig. 4.9A-C).


1A
DAPI
TLR10
Merge
Control
LPS 60m
LPS 90m
LPS 120m


1B
Gate: R2
Isotype control
RN1
Control
LPS 60 min
LPS 90 min
LPS 120 min
counts
Cell count
Fluorescent intensity

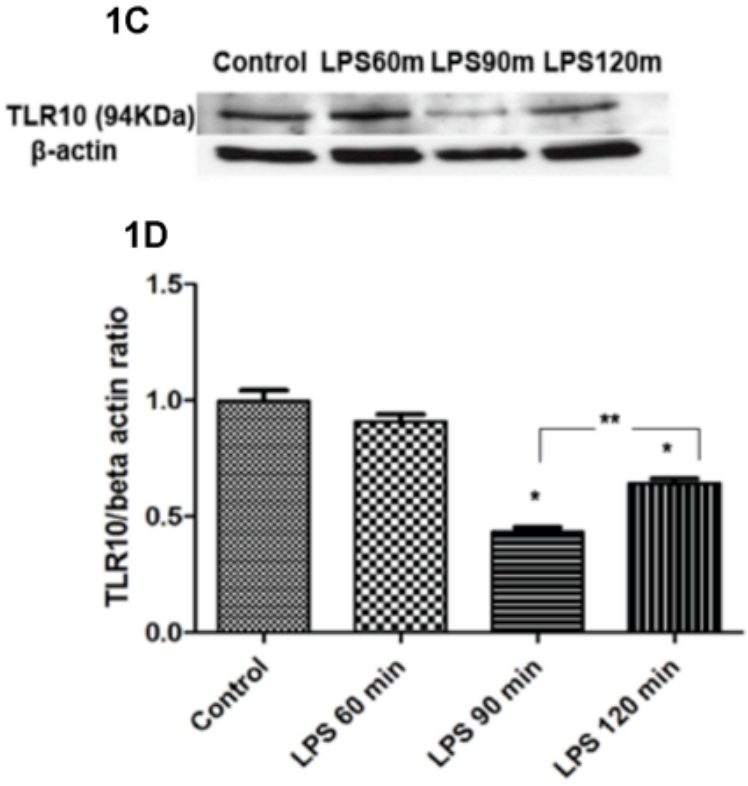
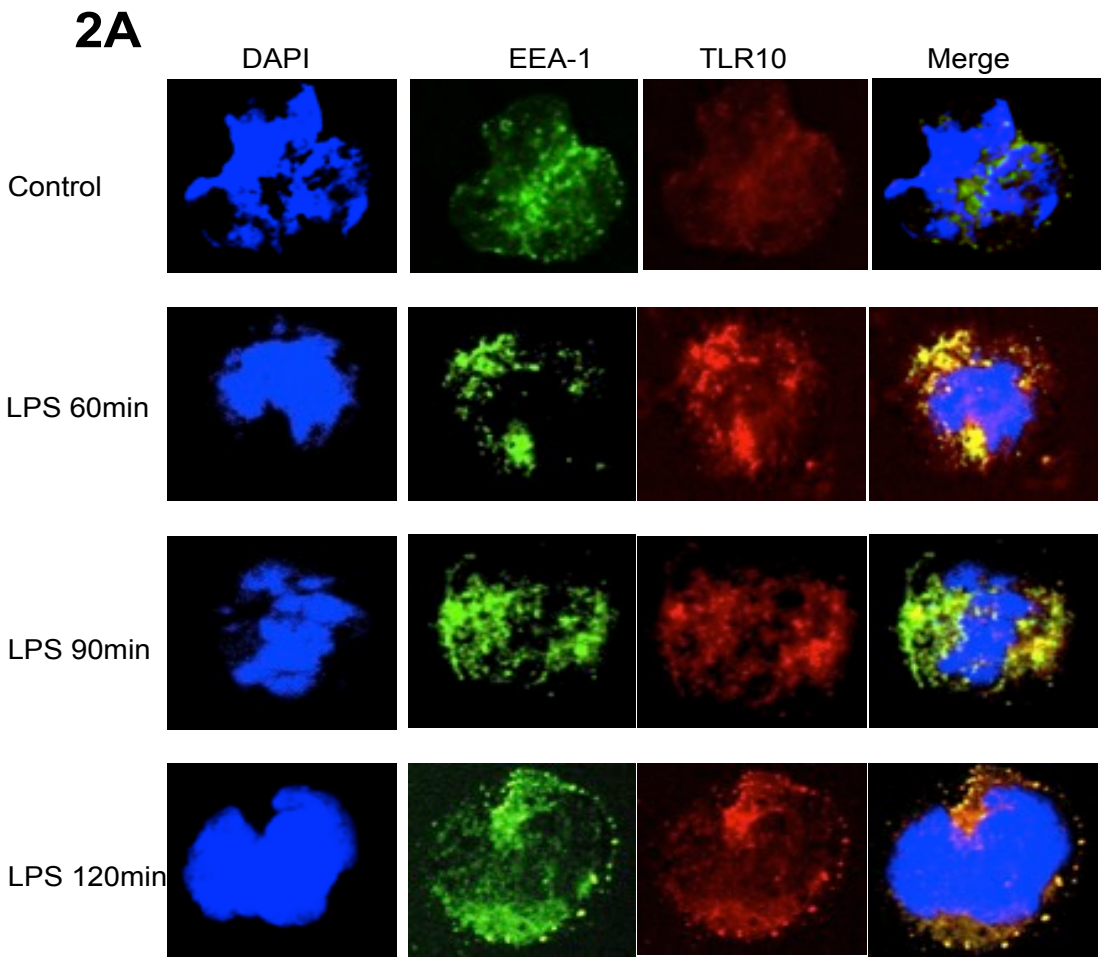
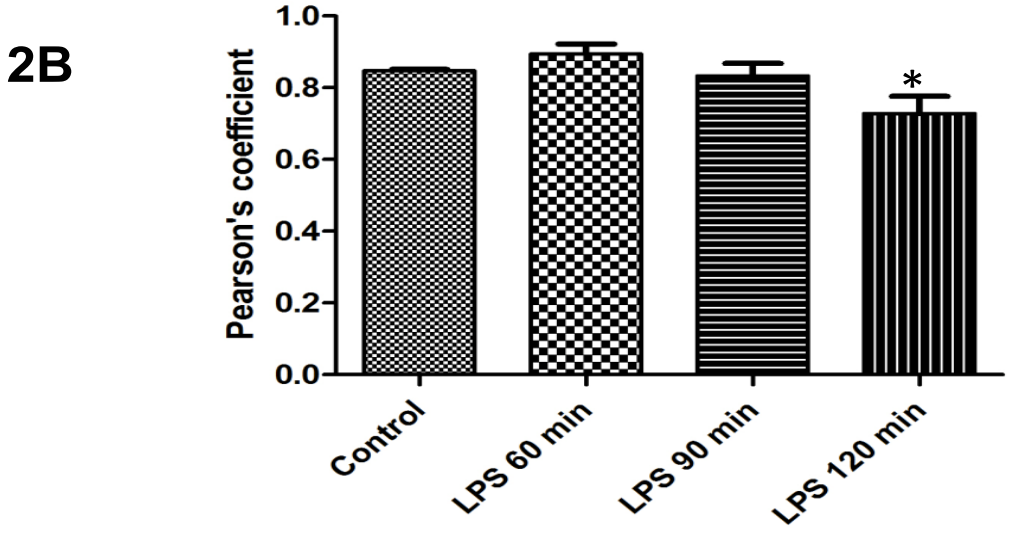


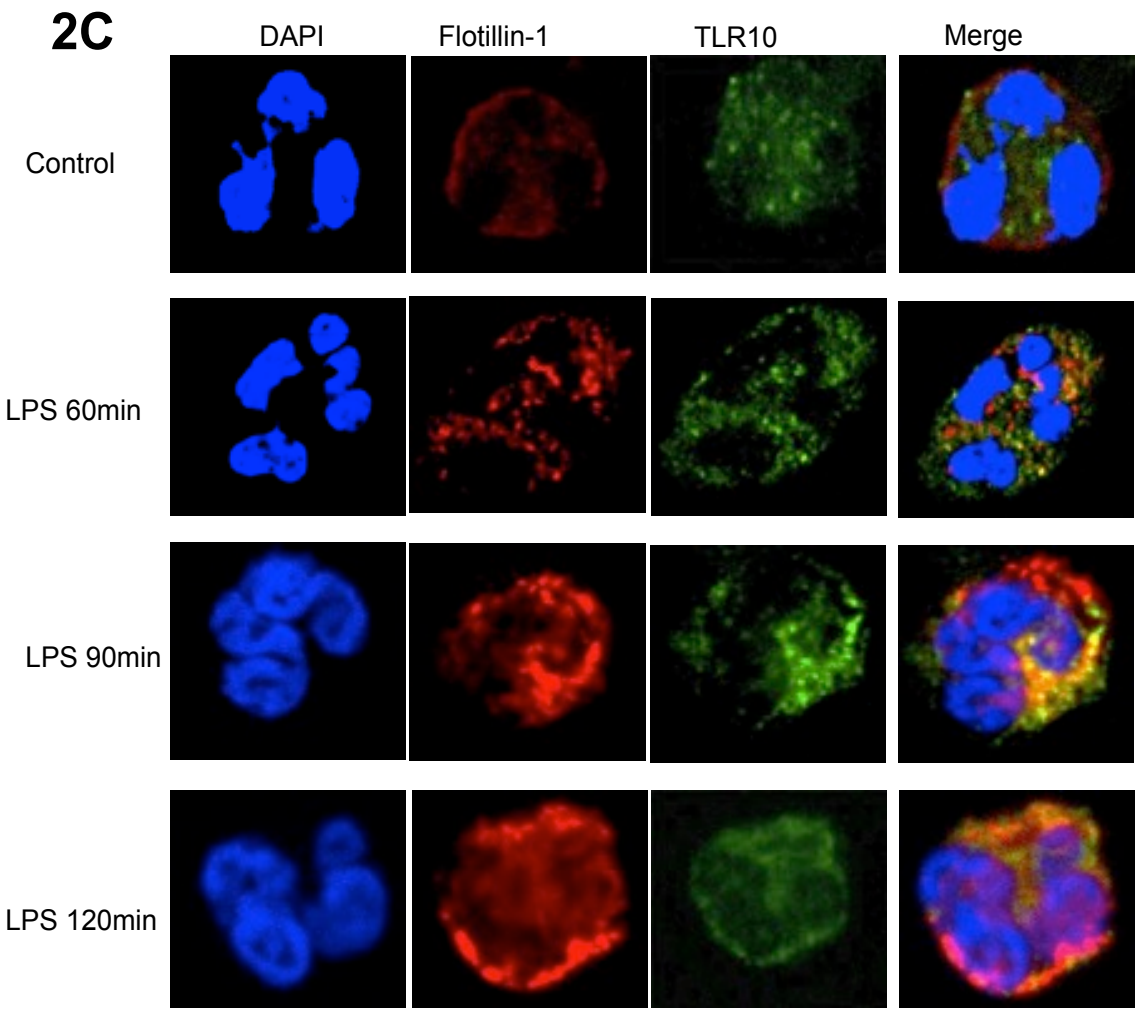
Figure 4. 1: LPS mediated temporal expression changes of TLR10

(A) Time dependent expression of TLR10 (in green) in human neutrophils (1 X 10^6). Nuclei stained in blue with DAPI. Neutrophils were treated with 1μg/ml LPS for 60, 90 and 120min (n=3). Imaged the temporal expression changes and localization of TLR10 expression during the treatment time in confocal microscopy. **(B)** Neutrophils stimulated with LPS (1μg/ml) for 60, 90 and 120min and stained with antibodies against TLR10 and isotype–matching antibody for flow cytometry analysis. FITC-TLR10 fluorescent spectrum shift was used to analyze TLR10 surface expression changes. **(C)** Immunoblots lysates of neutrophils (2 X 10^6). Cells were stimulated with LPS (1μg/ml) for 60min, 90 min and 120min. Molecular weight is depicted on the left side of the blots. β-actin showed in the lower panel referred as loading control. **(D)** Densitometry analysis showed

the down regulation of TLR10 expression in neutrophils treated with 90min and increased gene expression in LPS 120min (*p <0.05, compare with control; **p < 0.05, compare with LPS 60min). One representative experiment of three in the above experiments is shown.


2A
DAPI
EEA-1
TLR10
Merge
Control
LPS 60min
LPS 90min
LPS 120min


2B
Pearson's coefficient
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
LPS 60 min
LPS 90 min
LPS 120 min


2C
DAPI
Flotillin-1
TLR10
Merge
Control
LPS 60min
LPS 90min
LPS 120min

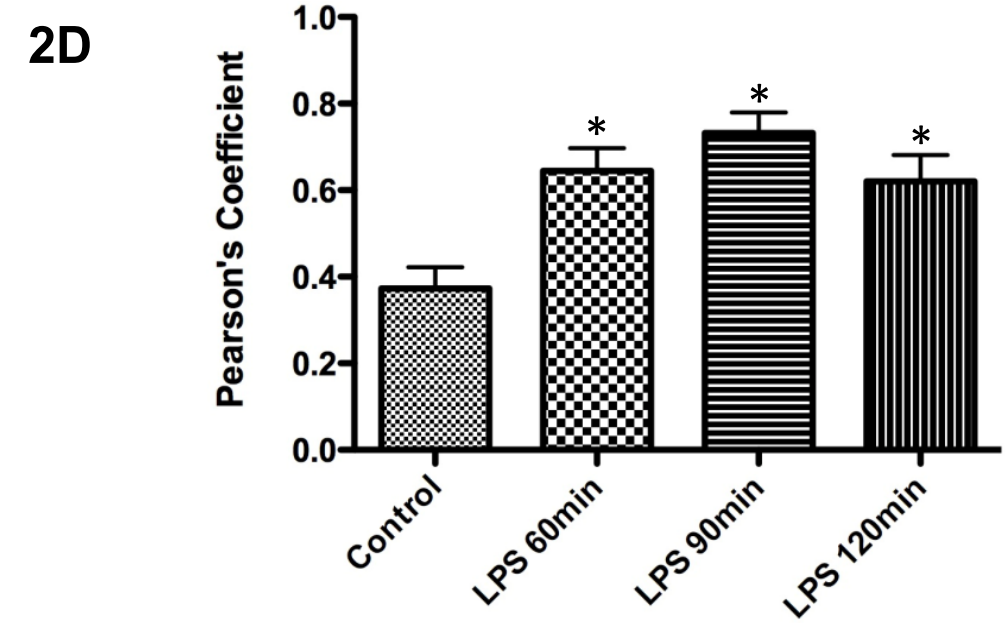
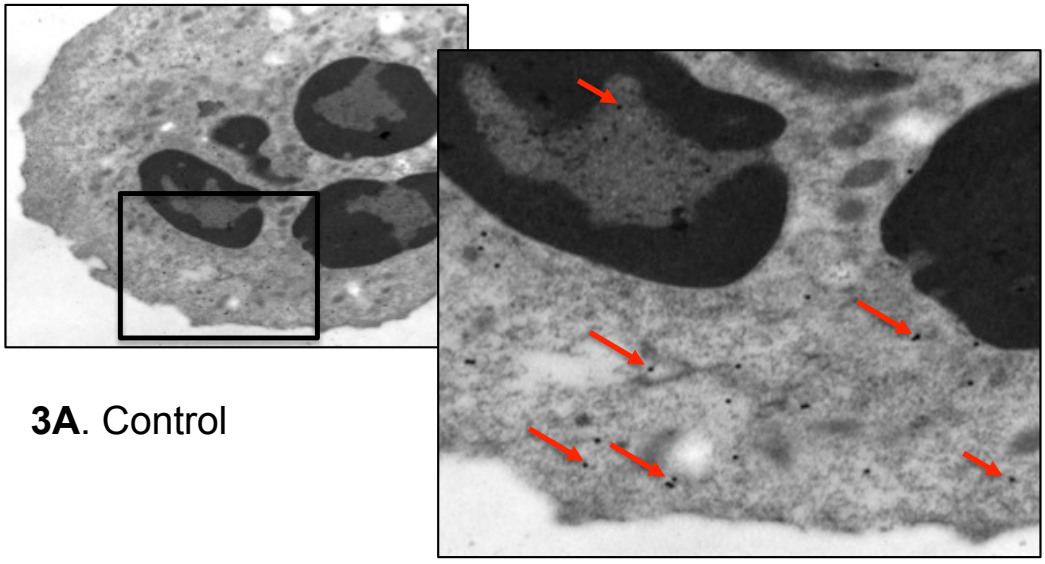
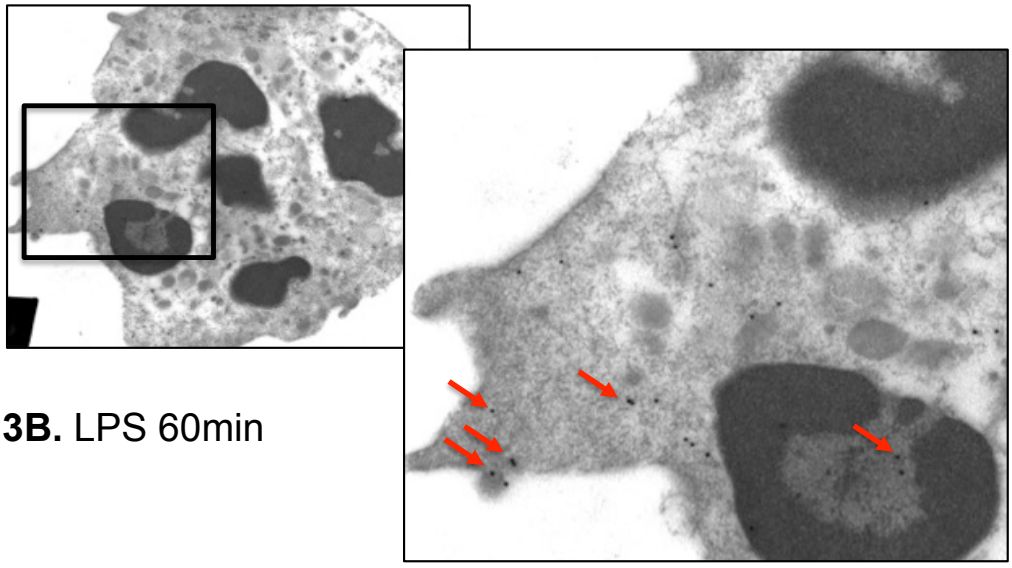

2D
Pearson's Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Control
LPS 60min
LPS 90min
LPS 120min

Figure 4. 2: Lipid raft mediated endocytosis of TLR10

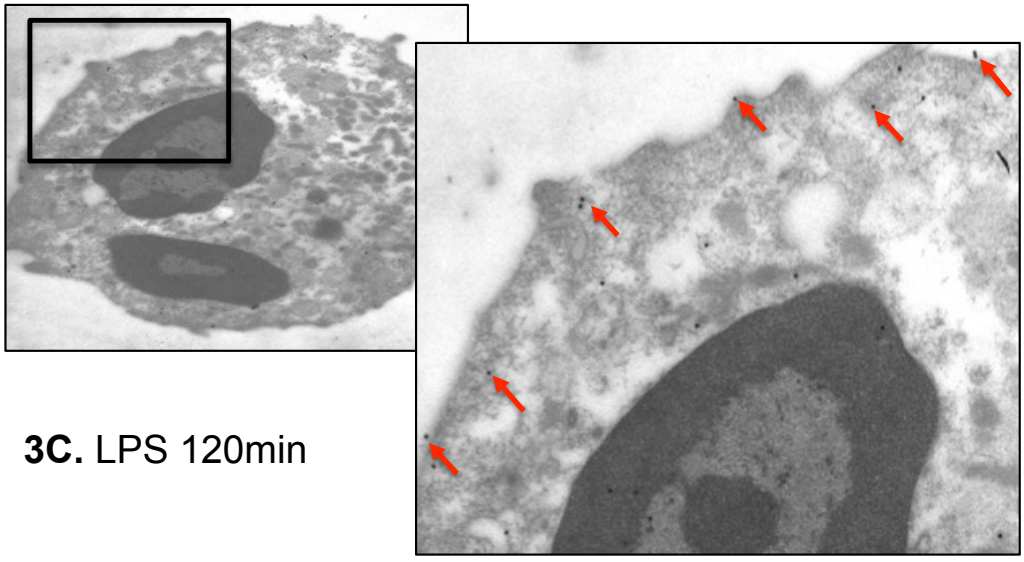
(A, B) Human neutrophils (1 X 10^6) adhered to FBS coated coverslips were activated by LPS (1μg/ml). Colocalization of TLR10 (red) and early endosomal antigen, EEA1 (green) are shown in merge panel. Treatment time points were 60min, 90min and 120min. Lower panel shows the graphical representation of quantification of colocalization in terms of Pearson's coefficient (*$p<0.05$), analyzed by Imaris 7.4 (Bitplane Inc., USA) using ImarisColoc module. **(C, D)** Colocalization of TLR10 (green) and flotillin-1, lipid raft marker (red) in LPS (1μg/ml) treated human neutrophils (1 X 10^6) adhered to FBS coated coverslips. Intact plasma membrane in control cells and membrane rearrangement during 60 – 120min was observed. Lower panel shows the graphical representation of quantification of degree of colocalization in terms of Pearson's coefficient (*$p<0.05$), analyzed by Imaris 7.4 (Bitplane Inc., USA) using ImarisColoc module. Results in **(A) - (D)** show representative data of three independent experiments.



3A. Control



3B. LPS 60min

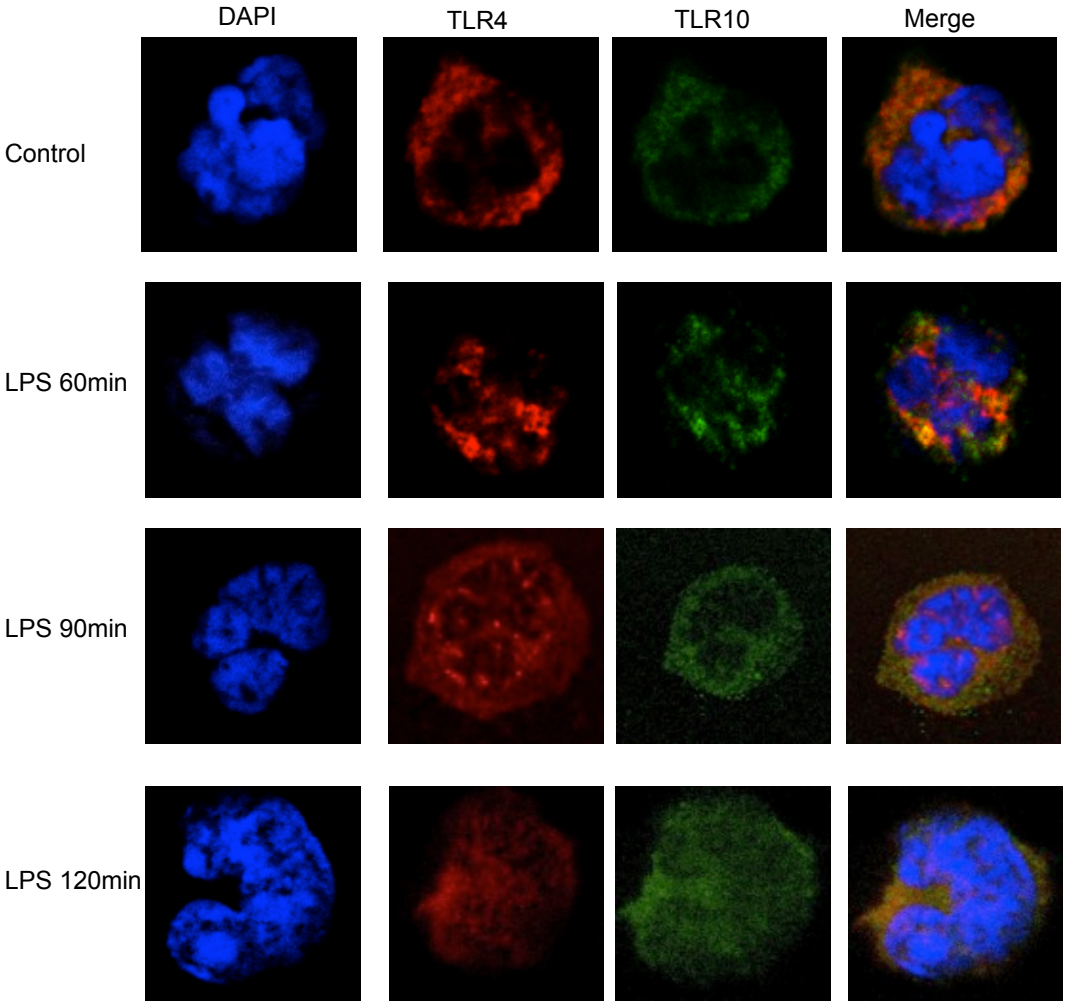


3C. LPS 120min

Figure 4. 3: Ultra-localization of TLR10 in human neutrophils

(A-C) Immuno-gold electron microscopy for TLR10 in human neutrophils shows the presence of TLR10 in nucleus, cytoplasm as well as in the plasma membrane (Red arrows). Note the TLR10 localization in pseudopodia of *E. coli* LPS (1µg/ml) activated neutrophils in Fig. B and C. Magnification. 13,000

4A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TLR4
TLR10
Merge
Control
LPS 60min
LPS 90min
LPS 120min

4B

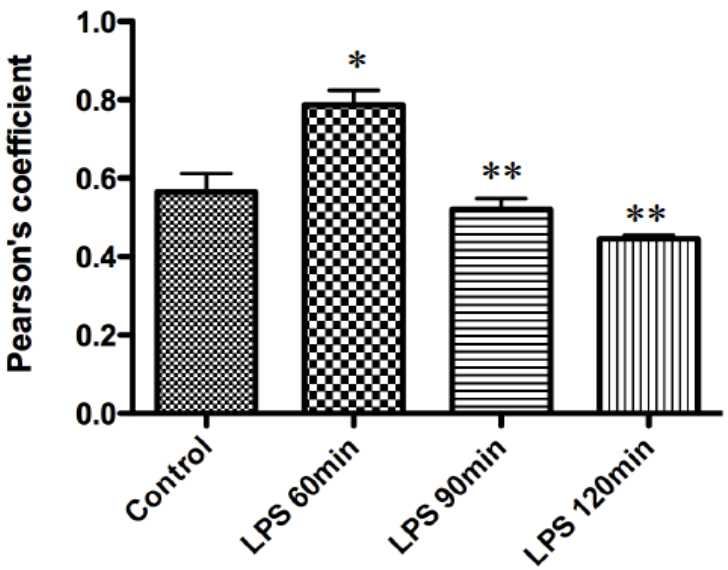

Pearson's coefficient
1.0
0.8
0.6
0.4
0.2
0.0
*
**
**
Control
LPS 60min
LPS 90min
LPS 120min

Figure 4. 4: TLR10 colocalized with TLR4 on LPS challenge

(A) Isolated human neutrophils (1 X 10^6) adhered on FBS coated coverslips were challenged with LPS (1μg/ml) for 60min, 90min and 120min and examined by confocal microscopy for the colocalization of TLR10 (in green) and TLR4 (in red). Merged channel indicate the overlapping signals from TLR10 and TLR4 along with nuclear stain DAPI. (B) Quantification of colocalization in terms of Pearson's coefficient analyzed by Imaris 7.4 (Bitplane Inc, USA) using ImarisColoc module. (*$p < 0.05$, compared with the control and **$p < 0.05$, compared with LPS 60min and all data shown in terms of mean $\pm$ SEM). One representative of three in the above different experiments is shown.

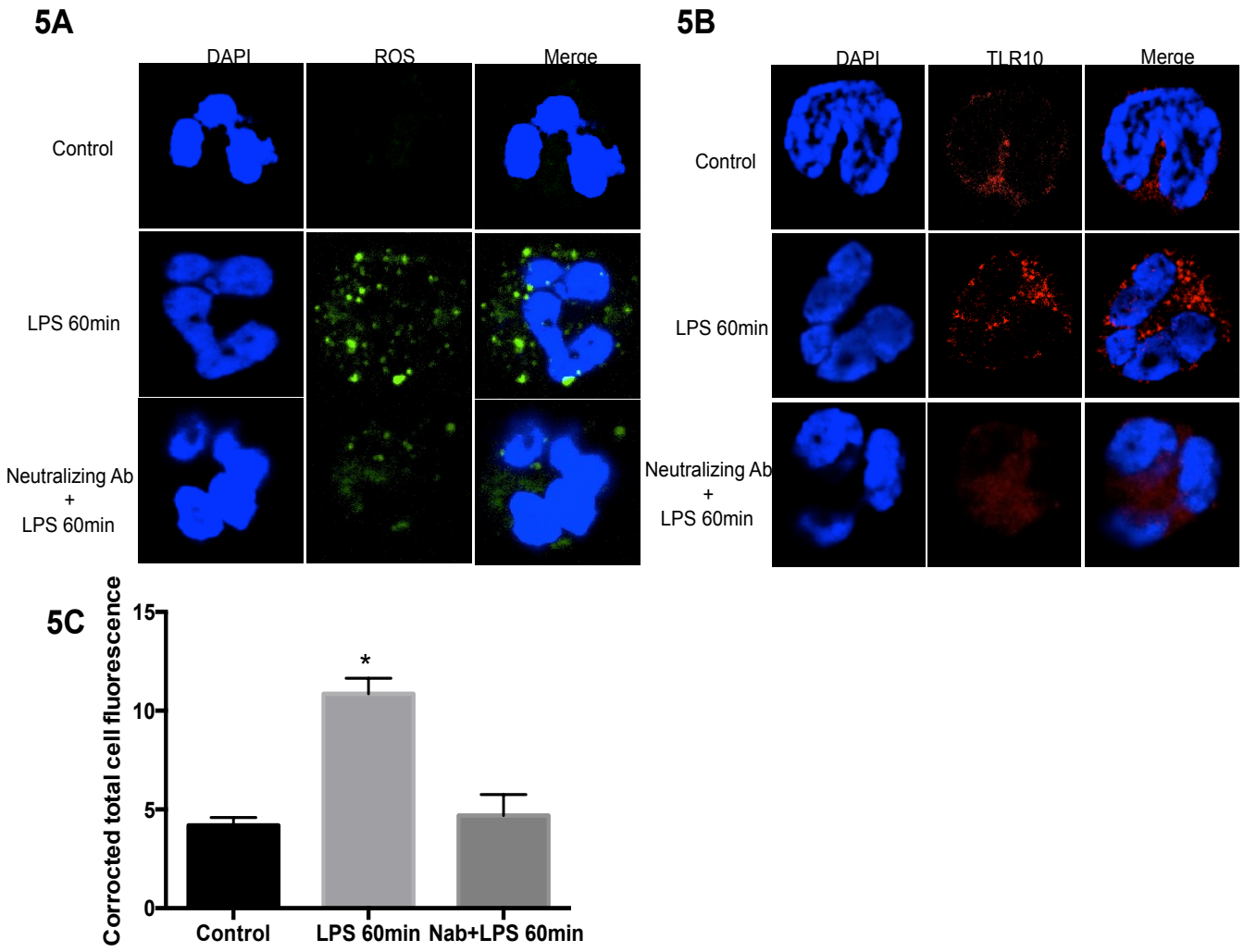
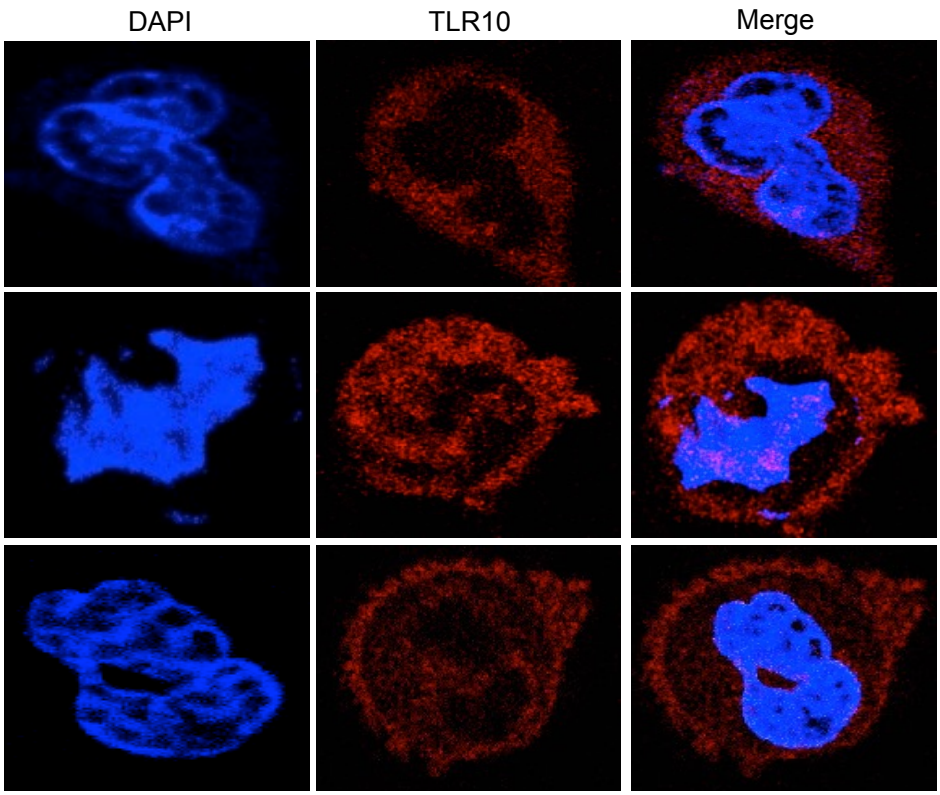


Figure 4. 5: TLR4 neutralization reduced TLR10 expression

(A) TLR4 neutralizing antibody (5µg/ml) co-incubated with isolated human neutrophils (1 X 10^6) for 1hr to inhibit bacterial LPS induced TLR4 signaling pathway. Cells were treated with bacterial LPS (1µg/ml) for 60min after TLR4 neutralization. ROS generation (in green) was measured as the marker for TLR4 neutralization and observed low ROS production in cells treated with neutralizing antibody. DAPI used to visualize the nuclei. **(B)** Isolated human neutrophils (1 X 10^6) treated for TLR4 neutralization and activated using bacterial LPS (1µg/ml); conditions as above. TLR10 (in red) was imaged using

confocal microscopy and merged image in the third column indicates the expression and cytoplasmic localization of TLR10 in control, LPS 60min treated as well as neutralizing antibody pretreated cells challenged with bacterial LPS for 60min. **(C)** Quantification of fluorescence in terms of corrected total cell fluorescence analyzed by Image J v1.47 (nih.gov, USA) using grey scale intensity analysis. (*$p < 0.05$, compared with the and all data shown in terms of mean $\pm$ SEM).

6A


DAPI
TLR10
Merge
Control
LPS 60min
FCCP
+
LPS 60min

6B

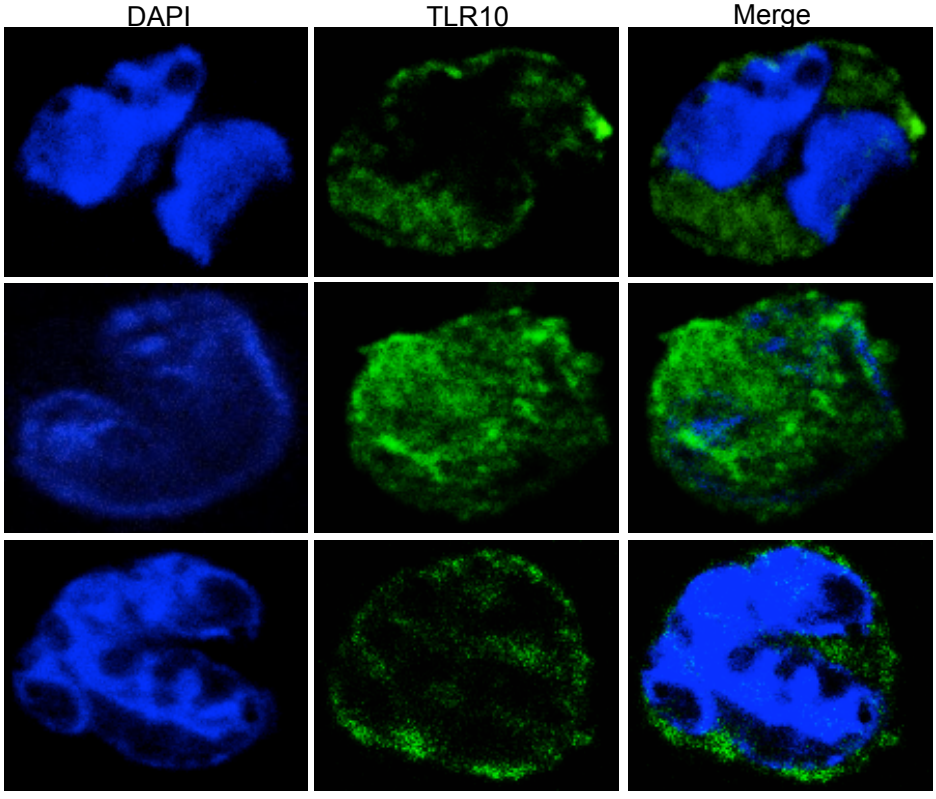

DAPI
TLR10
Merge
Control
LPS 60min
NF-kB inhibitor
+
LPS 60min

Figure 4. 6: ROS and NF-κB inhibition reduced TLR10 expression

(A) Human neutrophils (1 X 10^6) pretreated with Trifluorocarbonylcyanide Phenylhydrazone (Zablockaite et al., 2007), 5μg/ml, for 1hr at 37°C to deplete ROS production. Cells were measured for ROS generation (data not shown) to confirm the inhibitory activity of FCCP pretreatment. FCCP pretreated cells were challenged with bacterial LPS (1μg/ml) for 60min and imaged for TLR10 (in red) using confocal microscopy. Merged image shows TLR10 localization with nuclear stain, DAPI **(B)** Isolated human neutrophils (1 X 10^6) treated for NF-κB inhibitor and treated using bacterial LPS, conditions as above. TLR10 (in green) was imaged using confocal microscopy and merged image in the third column indicates the expression and cytoplasmic localization of TLR10 in control, LPS 60min treated as well as NF-κB inhibitor pretreated cells challenged with bacterial LPS for 60min.

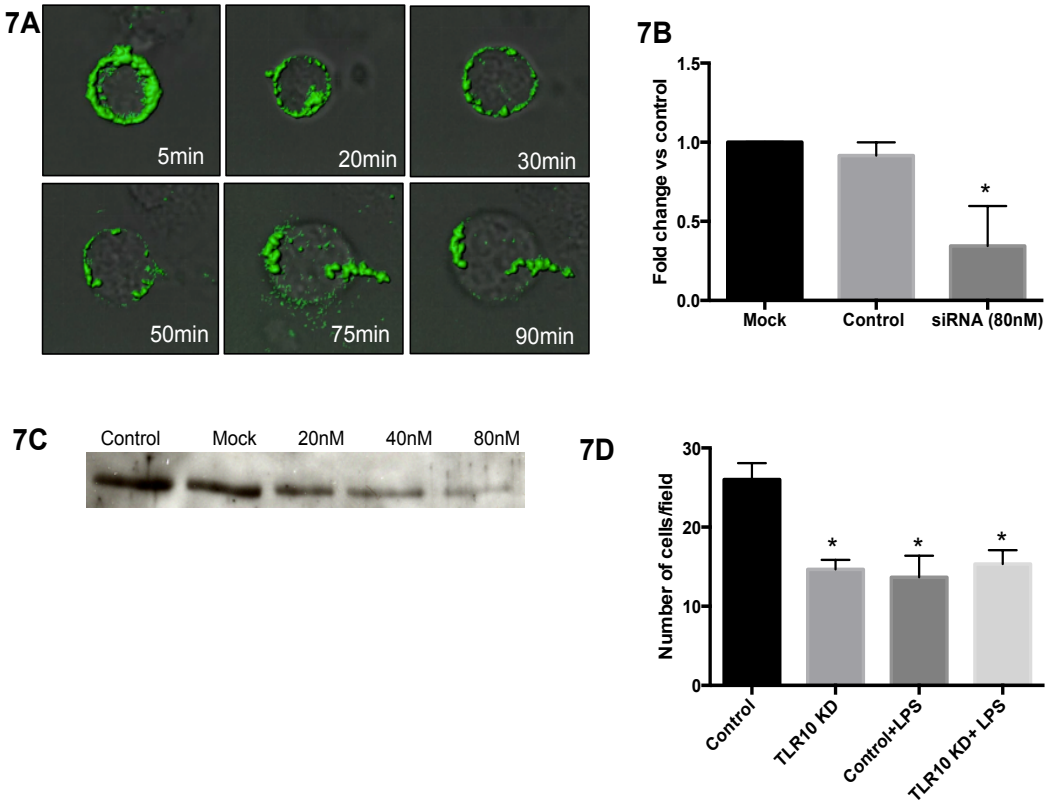


Figure 4. 7: Role of TLR10 in neutrophil chemotaxis

(A) Frames captured from live single cell imaging of human neutrophils. Neutrophils were labeled with anti-TLR10 antibody tagged with FITC (protocol as explained above) and imaged under Leica SP5 confocal microscope with heated stage (temperature maintained constantly at 37°C). Neutrophils were activated with concentration gradient of LPS (1µg/ml) attained by pipette tip diffusion and imaged for 100min. **(B)** HL-60 cell line was transfected with 80nM of TLR10 siRNA (sc-40272; Santa Cruz Biotechnology) using liposome-mediated transfection (Santa Cruz Biotechnology, 10410 Dallas, USA) after obtaining 80% confluence in culture. HL-60 differentiation in to neutrophils was achieved by the incubation with 1.3% DMSO for 5 days. Isolated mRNA used for quantitative real time PCR and calculated the fold change using ΔΔCt method. **(C)**

Immunoblots lysates of HL60 differentiated neutrophils (2 X 10^6). Cells were treated with 20nM, 40nM and 80nM of TLR10 siRNA and total protein isolate was hybridized against anti-TLR10 antibody. Molecular weight is depicted on the left side of the blots. **(D)** Chemotaxis experiment was performed using Boyden chamber with control, TLR10 knockdown, LPS treated (60 min; 1μg/ml) and TLR10 knockdown+ LPS treated (60min; 1μg/ml). Migrated cells from at least 5 different fields were counted and tallied. One representatives of two in the above experiments were shown.

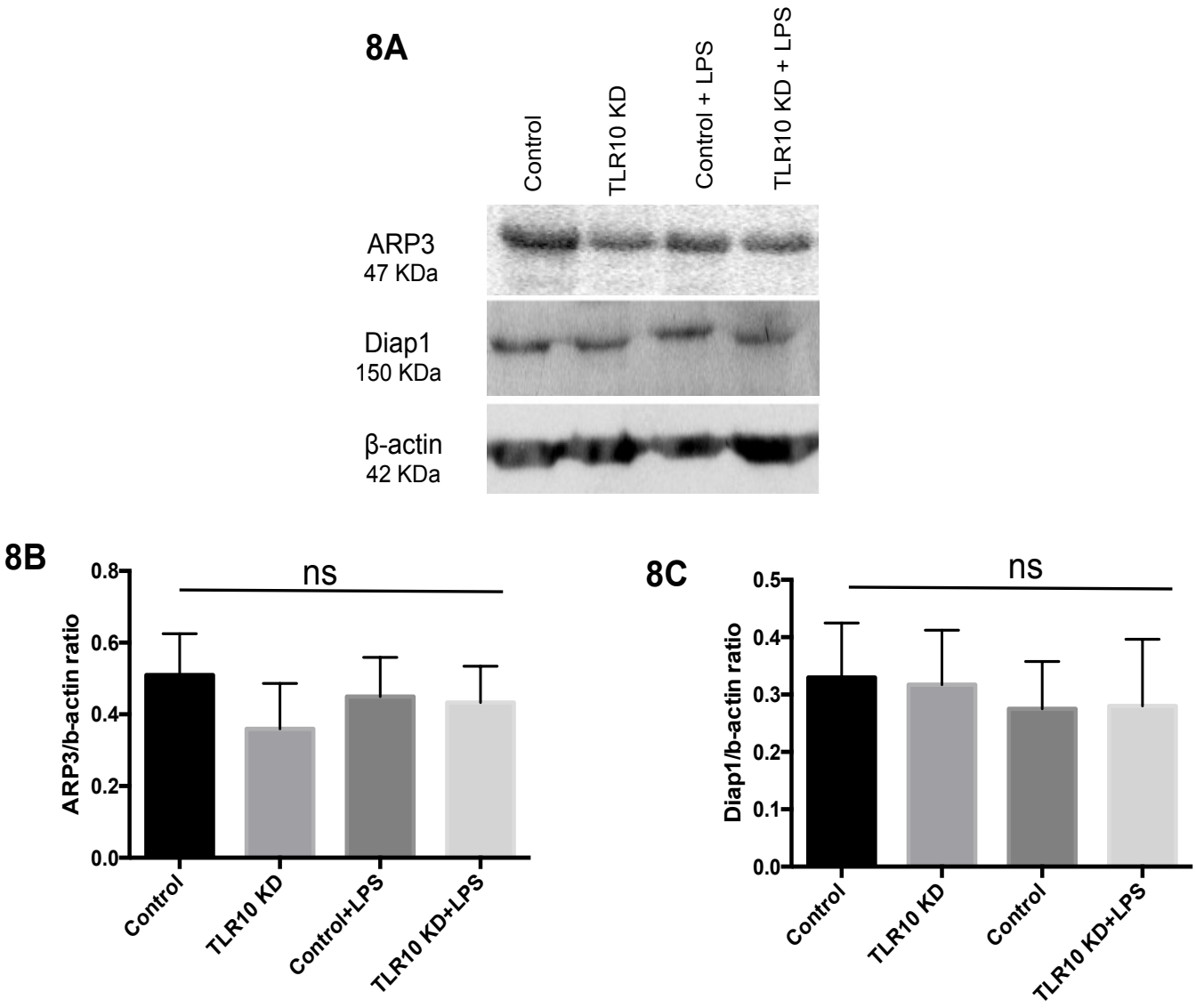
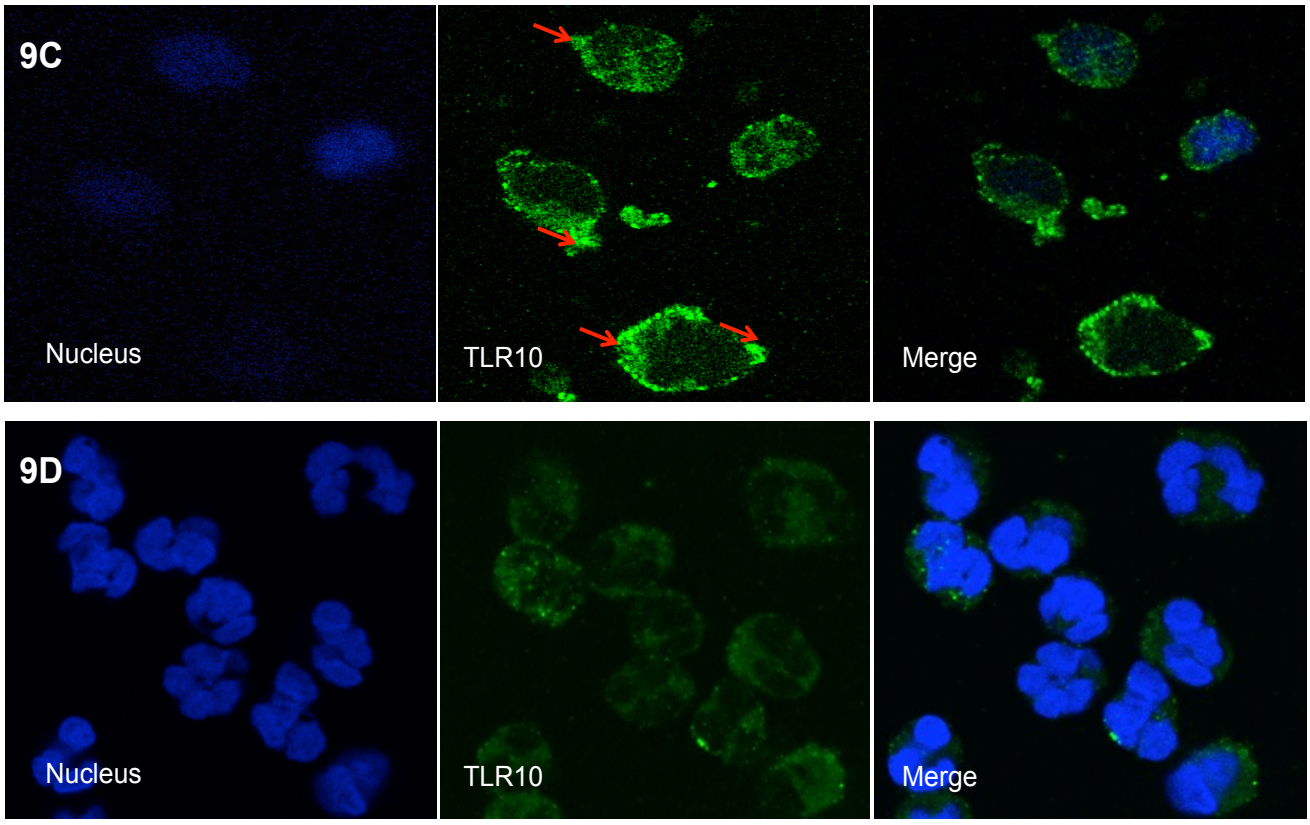


Figure 4. 8: TLR10 does not affect actin nucleation

(A) Immunoblots lysates of HL-60 derived neutrophils (2 X 10^6). TLR10 gene knockdown was performed as above and cells were stimulated with LPS (1µg/ml) for 60min. Actin nucleation proteins- ARP3 (1:500) and Diap1 (1:400) were detected in the blot corresponding to the specific molecular weight. Molecular weight is depicted on the

left side of the blots. β-actin showed in the lower panel referred as loading control. **(B & C)** Densitometry analysis showed no significant change in actin nucleation proteins.

9A
Nucleus
F-Actin
Merge
9B
Nucleus
F-Actin
Merge


9C
Nucleus
TLR10
Merge
9D
Nucleus
TLR10
Merge

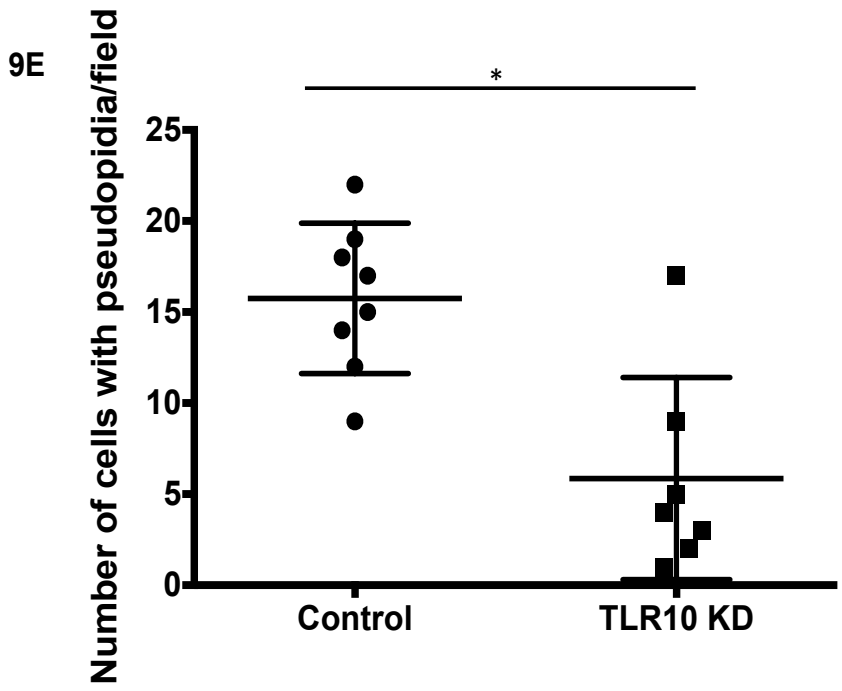


Figure 4. 9: TLR10 knockdown decreased formation of pseudopodia

Control (A) and TLR10 silenced (B) HL-60 derived neutrophils (1 X 10^6) were challenged with 1μM fMLP for 1min. F-actin (165nM) stained in green and nuclei (blue) stained with DAPI. Arrows (red) indicate pseudopodia formation. (C&D) Control and TLR10 knockdown HL-60 derived neutrophils (1 X 10^6) were challenged with 1μM fMLP for 1min. TLR10 stained in green and nuclei in blue stained with DAPI (E) Difference between the cells with pseudopodia in control and TLR10 knockdown groups. HL-60 derived neutrophils (1 X 10^6) were challenged with 1μM fMLP for 1min, imaged under confocal microscope and counted 8 different fields (double blinded counting).

4.5 Discussion

Although TLR10 was characterized in early 2000 (Chuang and Ulevitch, 2001), there are very little data on signaling pathway, ligand, and role of TLR10 in innate immunity. Hence, it is known as an "orphan receptor" of innate immunity. Functional TLR10 is not present in mice due to retroviral insertions in the TLR10 encoding gene thus precluding the use of mouse models to study the biology of TLR10. Neutrophils are the main effectors of innate immune system and first responders against a bacterial infection (Amulic et al., 2012). Hence, in this study, we provide novel data on the expression kinetics, localization dynamics in human neutrophils, and its role in TLR10 in chemotaxis.

First, we examined TLR10 expression and its regulation in neutrophils. Because the identity of TLR10 ligand is still not known, we used LPS to activate neutrophils via TLR4 pathway to understand the effect of cell activation on TLR10 expression. The TLR10 expression was reduced at 90min of LPS treatment and recovered to control values by 120min of the treatment. The new TLR10 gene transcription observed at 60min probably restored the TLR10 protein expression at 120min. Even more interesting is the rapid changes in plasma membrane expression of TLR10 through its endocytosis by activated neutrophils. Neutrophil activation led to changes in spatial and temporal expression of TLR10. Similar to TLR4 (Martha Triantafilou and Triantafilou, 2002), TLR10 endocytosis is also dependent on integrity of lipid rafts because the endocytosis was inhibited following disruption of lipid rafts. Taken together, the data show highly dynamic nature of TLR10 expression in activated neutrophils.

Second, we analyzed the mechanisms of regulation of TLR10 expression in activated neutrophils. Neutrophils upon stimulation with LPS produce ROS and translocate NF-κB into their nuclei (Neubauer et al., 2013). Earlier reports showed that ROS production is crucial in TLR4 dependent nuclear translocation of NF-κB (Asehnoune et al., 2004). ROS also contribute to LPS-TLR4 pathway induced upregulation of TLR2 in lung endothelial cells (Fan et al., 2003; Xiang et al., 2010). Kim and colleagues reported the increase in TLR10 mRNA expression with an increase in intracellular ROS (Kim et al., 2010). The data show that LPS-induced ROS production upregulates TLR10 expression in activated neutrophils because ROS depletion with FCCP inhibited increase in TLR10 expression. Our data also show that LPS alters TLR10 expression through TLR4 pathway as the effects were neutralized with a TLR4 neutralizing antibody, and thus, showing potential cross talk between TLR4 and TLR10. Lastly, the inhibition of nuclear translocation of NF-κB in neutrophils blocked LPS-induced changes in TLR10 expression to underscore the role of new gene transcription in regulation of TLR10 expression. Taken together, these data show that TLR4 pathway mediated production of ROS and nuclear translocation of NF-κB regulate the expression of TLR10 in activated neutrophils.

There are very limited data on the role of TLR10 in neutrophil biology. Our data show a novel role for TLR10 in neutrophil chemotaxis, one of the primal functions of neutrophils. Even though TLRs are involved in PAMPs detection and regulation of proinflammatory cytokine secretion, which promotes leukocyte recruitment to the site of injury, many reports argue for the role of activated TLR in inhibiting leukocyte chemotaxis (Khan et al., 2005; Sabroe et al., 2005; Alves-Filho et al., 2009; Yi et al.,

2012a). However, there are contradictory data stating the modulatory effects of TLR activation on neutrophil chemotaxis. Fan and colleagues reported increased chemotaxis towards MIP-2 in LPS-challenged neutrophils. LPS induced TLR4 activation downregulated G-protein-coupled receptor kinases (GRK) 2 and 5, which in turn desensitized chemokine receptors on neutrophils, resulted in enhancing the chemotactic response (Fan and Malik, 2003b; Hayashi et al., 2003; Aomatsu et al., 2008a). Meanwhile, Filho and co-workers showed TLR2 signaling induced with LTA upregulated GRK2 expression and inhibited neutrophil chemotaxis (Alves-Filho et al., 2009). My video-microscopy of live neutrophils and immuno-electron microscopy of fixed and embedded neutrophils showed aggregation of TLR10 on the leading edge of activated neutrophils. The TLR10 knockdown results in reduced chemotaxis of neutrophils towards fMLP along with a reduction in the numbers of plasma membrane pseudopods. Previous studies have shown that TLRs regulate actin polymerization, phagocytosis and chemotaxis by modulating MAPK, Cdc42, and Rac1 pathways (Doyle et al., 2004; Kong and Ge, 2008; Alves-Filho et al., 2009). We further attempted to address the role TLR10 in ARP3 and Diap1 proteins expression, which involve in actin nucleation, one of the steps in pseudopod formation. However, the western blot data showed no differences in the expression of these proteins in normal neutrophils or those subjected to TLR10 knockdown. While there is need for further experiment to understand the mechanisms that regulate reduction in membrane pseudopods following in TLR10 knockdown in neutrophils, the role of TLR10 in neutrophil chemotaxis is novel nevertheless.

Genetic polymorphisms of TLR10 have been reported in a number of inflammatory diseases such as asthma, Crohn's disease, prostate cancer etc. (Lazarus et al., 2004; Chen et al., 2007) indicating its functional role in immune system and the molecular mechanisms need to be explored. However, our data are the first to show detailed expression, dynamics, ultrastructural localization, and functional roles of TLR10 in bacterial LPS induced innate immune response. It is plausible that TLR10 contributes a key regulatory role in innate immunity and this basic data opens an intriguing area for future studies.

CHAPTER 5: ROLE OF TOLL-LIKE RECEPTOR 10 IN INNATE IMMUNE RESPONSE AGAINST *Streptococcus pneumoniae* INFECTION IN HUMAN MACROPHAGES

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Running title: Role of TLR10 in *S. pneumoniae* infection

Author contributions: Y.B, and B.S conceived the idea and R.K provided the human samples and directions for immunohistological analysis and Y.B performed research. Y.B and B.S wrote the manuscript.

Keywords: TLR10, *S. pneumoniae*, macrophages, TNF-α, interleukins, TLR10 siRNA

5.1 Abstract

Toll-like Receptors (TLRs), known as "Gate Keepers" of innate immunity, are evolutionarily conserved receptors to recognize pathogen-associated molecular patterns. TLRs play a critical role in innate immunity and in early response against invading pathogens. Even though, TLRs have been widely studied, very little is known about the expression and function of TLR10. Since TLR10 is a pseudogene in mice, there are no successful rodent animal models to study the function of TLR10 through genetic manipulations. Till date, no data are available on tissue and cell specific expression of TLR10 in normal and inflamed human lungs as well as in primary immune cells such as macrophages. *S. pneumoniae* are Gram-positive, alpha-hemolytic and major causative agents of pneumonia, ear infections, sinus infections, meningitis, and bacteremia. We examined the role of TLR10 in innate immune response to *S. pneumoniae* infection in U937 cell line derived human macrophages. We found a significant increase in TLR10 mRNA expression in macrophages challenged with *S. pneumoniae* (10^7 cfu for 6hr). TLR10 knockdown resulted in significant reduction in expression of IL-1β, IL-8, IL-17 and TNF-α but didn't affect IL-10 expression. TLR10 knockdown also reduced nuclear translocation of NF-κB during macrophages challenged with *S. pneumoniae*. However, TLR10 knockdown had no effect on the phagocytosis of the bacteria by the macrophages. TLR10 expression in vascular endothelium in normal and inflamed human lungs was confirmed by immunohistochemistry. Taken together, our data show that TLR10 is expressed in human lung vasculature and macrophages, and regulates expression of cytokines in macrophages infected with *S. pneumoniae*.

5.2 Introduction

Streptococcus pneumoniae is a gram positive diplococcus human pathogen responsible for approximately 1.2 million infant deaths per year around the globe (O'Brien et al., 2009; PHAC, 2011). *S. pneumoniae* colonizes the upper respiratory tract and causes lethal meningitis, pneumonia, and sepsis (Chiavolini et al., 2008; Bogaert et al., 2009). Pneumolysin (PLY), an exotoxin with cytotoxic activity, is the important virulence factor of *S. pneumoniae*. PLY of most pneumococcal strains is cytotoxic to mammalian cells by forming large pores into membranes (Sutherland and Martin, 2007).

The first recognition of invading pathogens such as *S. pneumoniae* by the host is mediated by pattern recognition receptors (PRRs) of the immune system. So far 10 TLRs have been identified in humans namely TLR1 to 10 (Takeda et al., 2003). TLRs are highly conserved PRRs, which detect specific pathogen associated molecular patterns (PAMPs), initiate a cascade of reactions and lead to the activation of proinflammatory cytokine production to remove the invading pathogen. Upon binding to their specific ligands, TLRs trigger the innate inflammatory response through the activation of series of adaptor molecules (Beutler, 2009). Previous data suggest TLR 2, 4 and 9 play important roles in modulating bacterial susceptibility, higher bacterial loads, and heightened upper respiratory tract cell apoptosis during *S. pneumoniae* infection (Koppe et al., 2012). Increasing evidence indicates that activation of innate immune system is an essential criterion for the induction of adaptive immunity. Based on the protein sequences and pathogen associated patterns they detect, TLRs can be further categorized into two

subfamilies: TLR1, 2 and 6 subfamily recognizes lipids, TLR7, 8 and 9 subfamily recognize nucleic acids (Akira et al., 2006).

TLR10 is recently identified TLR family protein and is non-functional in mice due to exonal retroviral insertions (Chuang and Ulevitch, 2001). TLR10 is functional in humans and is expressed by immune cells like dendritic cells, macrophages, and B cells. TLR10 is present in the lungs of many species including pig, dog, cattle, mice and chicken (Balachandran et al., 2015). Sequence analysis confirmed that TLR10 aligns in the same locus with TLR1 and 6. The ligand of TLR10 is still unknown. TLR10 forms homodimers with each other as well as heterodimers with TLR1 and TLR2 (Hasan et al., 2005). TLR1-TL6-TLR10 gene cluster polymorphism can increase disease susceptibility for prostate cancer (Chen et al., 2007). TLR10 polymorphisms were observed in patients with clinical symptoms of Crohn's disease, papillary thyroid carcinoma, urothelial bladder cancer, Turkish rheumatoid arthritis, and asthma (Lazarus et al., 2004; Etem et al., 2011; Guirado et al., 2012; Morgan et al., 2012; Kim et al., 2013). While lack of a recognized ligand for TLR10 hinders many functional studies, it is known that hypoxia upregulates TLR10 mRNA in human monocytic cell line, THP-1, through activation of reactive oxygen species (ROS), NF-κB, and AP-1 pathways. Luciferase binding assays indicated a potential NF-κB binding site in TLR10 gene sequence (Halliwell et al., 1999; Kim et al., 2010). Viral infection in dendritic cells caused TLR10 upregulation and activation of interferon 1 signaling pathway (Hertzog et al., 2003). The data reported in this thesis (Chapter 4) also show the regulation of TLR10 expression in neutrophils via ROS and NF-κB pathways following ligation of TLR4 with LPS. Currently, there are no data on the interaction of TLR10 with specific bacterial pathogens.

Although the interaction between innate immune receptors and bacterial pathogens has been extensively studied, little is known about the role of TLR10 in innate immune reaction against gram-positive bacteria. Therefore, first we examined the expression of TLR10 in the human lung. Following that we studied the role of TLR10 in responses to *S. pneumoniae* infection by U937 derived macrophage cells. Our data suggests that TLR10 is expressed in human lungs including epithelial cells and macrophages, the expression of TLR10 is upregulated during *S. pneumoniae* infection and TLR10 regulates the production of IL-1β, IL-8, IL-17 and TNF-α.

5.3 Materials and methods

5.3.1 Cell culture

U937 is a human histiocytic lymphoma cell line purchased from ATCC (CRL-1593.2), maintained in RPMI-1640 media (ATCC) with 10% FBS (Life Technologies), and supplemented with 10µg/ml penicillin and streptomycin (Sigma Aldrich). The cells were incubated at 37°C with 5% CO_2. Differentiation of U937 cells into macrophage lineage was achieved in 48hr using 20nM Phorbol-12-Myristate-13-Acetate (PMA; Sigma Aldrich). Invasive live *S. pneumoniae* (See below) used to challenge the cells.

5.3.2 Bacterial culture and infection

S. pneumoniae (Klein Chester ATCC 6303) strain with type 3 antigenic properties was commercially purchased from ATCC (Manassas, USA). Bacteria were cultured in brain heart infusion broth (Sigma Aldrich) with 1% glucose at 37°C, shaking at 130 g for

24hr supplemented with 5% CO_2. Bacterial dilution and count were estimated using McFarland standard dilution comparison method. In brief, we compared 24hr grown *S. pneumoniae* culture to McFarland standard 5 (turbidity scale-5; OD equivalent- 0.65) and allowed the culture to reach the known OD of McFarland standard 5. We made serial dilutions (10^{-1}- 10^{-7}) of the bacterial culture (OD=0.65) and plated in Columbia blood agar and incubated over night to calculate the colony forming units corresponding to each dilution. For infecting the macrophages, desired dilution of *S. pneumoniae* culture in log phase was washed with PBS and added to U937 derived macrophage cells with antibiotic free media.

5.3.3 Small interfering RNA transfection to knockdown TLR10 gene expression

After attaining 80% confluence, U937 derived macrophages were transfected with TLR10 siRNA using commercially available liposomal transfection system (Santa Cruz Biotechnology, 10410 Dallas, USA). In brief, 80nM of TLR10 siRNA (sc-40272; Santa Cruz Biotechnology) was incubated with transfection reagent for 45min to form transfection reagent-siRNA complex and added this mixture to the cells washed with transfection medium. Cells were incubated for 8hr in the transfection mixture followed by a wash with and incubation in RPMI-1640 medium for 48hr.

5.3.4 Quantitative real time PCR

Total RNA was isolated from the macrophage cell line incubated with *S. pneumoniae* using Qiagen RNeasy Mini kit followed by the treatment with RNase free DNase (Qiagen, Ontario, Canada) according to the manufacturer's instructions. RNA was

quantified by Nanodrop method and cDNA was prepared using Quantitect Reverse Transcription Kit (Qiagen, Ontario, Canada). Quantitative real time PCR was performed to quantify transcriptional level expression of IL-1, IL-8, IL-17, TNF-α and IL-10 using Stratagene MX3005P PCR instrument and brilliant SYBR Green QPCR kit (Agilent Technologies, Santa Clara, USA) was used for the reaction. Primers were obtained from Invitrogen (Burlington, Canada) and sequences are listed as in Table. 1. ROX was used as reference dye for the PCR reaction. Specificity of the reaction was measured with non-template and no-reverse transcriptase controls and analysis of melting curves. GAPDH was used for normalization of the expression.

5.3.5 Confocal microscopy for TLR10 and NF-κB

U937 derived macrophages were cultured in 37°C and 5% CO_2 in RPMI-1640 with 10% FBS. Live *S. pneumoniae* cells (10^7 cfu/well) were inoculated for 6hr and incubated as described above. Four percent paraformaldehyde was used as a fixative and cells were permeabilized with 0.01% TritonX 100. Nonspecific antigens were blocked by 5% BSA. Fcγ blocking was performed as previously described (Sedlmayr et al., 2001). TLR10 (1:250) and anti-NF-κB p65 (1:200) primary antibodies incubated for 1hr at room temperature. Fluorescent-labeled secondary antibody incubation was done for 30 min at room temperature. Cells were mounted in medium containing DAPI and left overnight for proper conditioning of the slides for imaging. *S. pneumoniae* were labeled with Oregon green (Maximum excitation at 511nm, maximum emission at 530nm). Confocal microscopy was performed in Leica TCS SP5 system (Leica Microsystems, Germany) with 63X magnification under oil for acquisition.

5.3.6 Immunohistochemistry for TLR10 in human lungs

Immunohistochemistry was performed on sections obtained from paraffin embedded lung tissue samples from control (n=7) and asthmatic (n=6) humans. The sections were deparaffinized and rehydrated followed by incubation with 5% hydrogen peroxidase to neutralize endogenous peroxidase. The antigen retrieval step included treatment with pepsin (2mg/ml in 0.01N HCL). The sections were exposed to BSA (1% BSA in 1X PBS) to block non-specific binding of immunoglobulins. We used von Willebrand factor (vWF; 1: 750) as a positive control because it is constitutively expressed in vascular endothelium (Itaru et al., 2002) whereas staining without primary antibody and staining with isotype matching antibody were used as negative control. Lung sections were stained for TLR10 (1: 150). The color development was performed with Vector color developing agents after HRP conjugated secondary antibody incubation (1: 150, Dako Laboratories, Denmark).

5.3.7 Statistical analysis

Results were represented as mean $\pm$ SEM and analyzed using Student t- test. A p value ≤ 0.05 was considered as statistically significant. All analyses were performed in GraphPad Prism v6.0 (GraphPad Software Inc., La Jolla, USA).

5.4 Results

5.4.1 TLR10 is expressed in U937 derived human macrophages and its expression is upregulated by *S. pneumoniae*

We investigated the effect of size of live *S. pneumoniae* inoculum (10^5, 10^6, 10^7 and 10^8 cfu) and time (3hr, 6hr, 12hr and 24hr) of incubation on TLR10 expression on U937 derived human macrophages. TLR10 mRNA expression was significantly upregulated with 10^7 and 10^8 cfu live *S. pneumoniae* compared to control after 6hr of culture with the macrophage cell line (Fig. 1A). Highest levels of TLR10 mRNA expression were recorded in macrophages treated with 10^7 cfu *S. pneumoniae* for 6hr but significantly higher TLR10 mRNA was observed at 2, 6 and 12hrs of infection (Fig. 1B). TLR10 protein expression and localization after infection with live *S. pneumoniae* was also determined by confocal microscopy. TLR10 was expressed in the cytoplasm of control macrophages. However, 6hr of incubation with 10^7 cfu of *S. pneumoniae* induced more intense and diffuse TLR10 expression in the cytoplasm and the nucleus. Corrected total cell fluorescence showed higher fluorescence intensity of TLR10 staining in macrophages incubated with *S. pneumoniae* for 6hr compared to the control macrophages (Fig. 1C and 1D).

5.4.2 TLR10 regulates cytokine induction in response to *S. pneumoniae* infection

We next investigated the effect of TLR10 on selected proinflammatory (IL-1β, IL-8, IL-17 and TNF-α) and anti-inflammatory (IL-10) cytokines after 6hr of *S. pneumoniae* challenge. The knockdown of the TLR10 protein expression in macrophages was achieved by using 80nM TLR10 siRNA (Fig. 2A) and confirmed with confocal microscopy (Fig. 2B). The knockdown of TLR10 in macrophages infected with *S. pneumoniae* significantly reduced the expression of IL-1β (61%), IL-8 (62.8%), IL-17 (47%), and TNF-α (32%) mRNA compared with control macrophages (Fig. 3A-D). IL-10 mRNA expression was not affected by TLR10 knockdown (Fig. 3E).

5.4.3 TLR10 does not affect phagocytosis of *S. pneumoniae*

In order to investigate whether TLR10 silencing influences the phagocytic activity of macrophages, which may have subsequently affected the production of inflammatory mediators, we incubated the control macrophages as well as those following TLR10 knockdown with fluorescent-labeled live *S. pneumoniae* for 6hr. Cells were imaged under confocal microscopy and counted number of bacterial cell per macrophages. Analysis of phagocytic activity showed no significant difference between control and TLR10 knockdown macrophages (Fig. 4A-B).

5.4.4 TLR10 knockdown reduced nuclear translocation of NF-κB

To investigate the mechanisms through which TLR10 regulates cytokine production, we examined the nuclear translocation of transcription factor NF-κB. TLR10

knockdown reduced the nuclear translocation of NF-κB in *S. pneumoniae* challenged macrophages compared to the control suggesting that NF-κB might be the effector molecule of TLR10 receptor activation (Fig. 5A). Western blots on the nuclear extracts from the above experiment showed reduced nuclear localization of NF-κB in macrophages (Fig. 5B) in macrophage cells that had their TLR10 knocked down compared to the controls.

5.4.5 Inflammation increased the expression of TLR10 in human lung

When asthma patients acquire viral infections, which, in turn, trigger the asthmatic response, they may develop subsequent bacterial infections, mainly by *S. pneumoniae* (Otero et al., 2013). Therefore, we analyzed the TLR10 expression in normal and asthmatic lungs. Lung sections stained with vWF showed positive staining in the vascular endothelium (Fig. 6A) while replacement of the primary antibody with an isotype-matched antibody (Fig. 6B), or omission of primary antibody altogether (data not shown) resulted in absence of reaction. TLR10 expression was observed in endothelium and sub-epithelial area of bronchiole (Fig. 6C) of normal human lung. Asthmatic lungs showed elevated expression in both endothelium and alveolar septa (Fig. 6D-F). The data indicate that TLR10 expression is changed during infection and chronic inflammation suggesting important role as innate immune receptor.

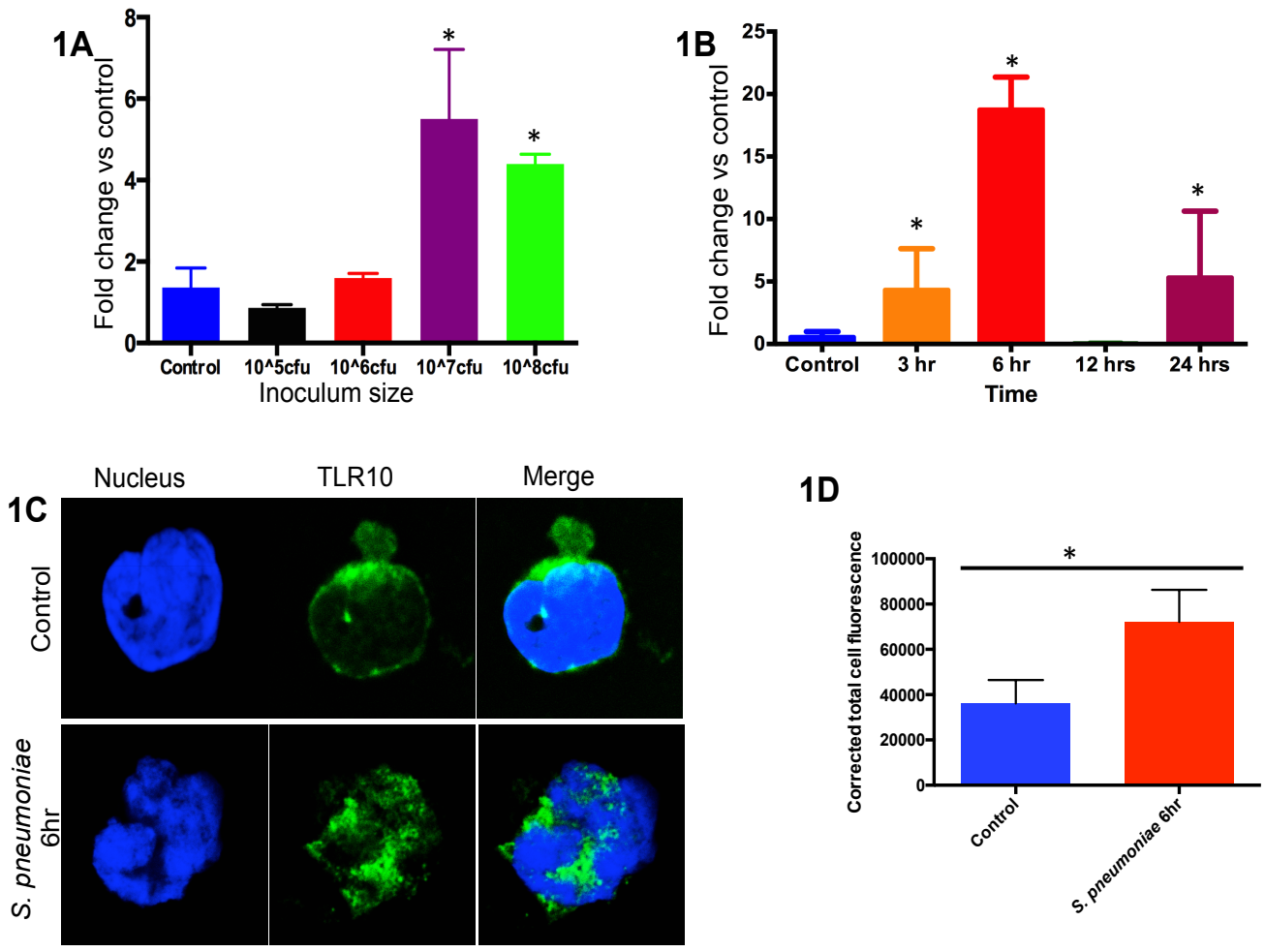
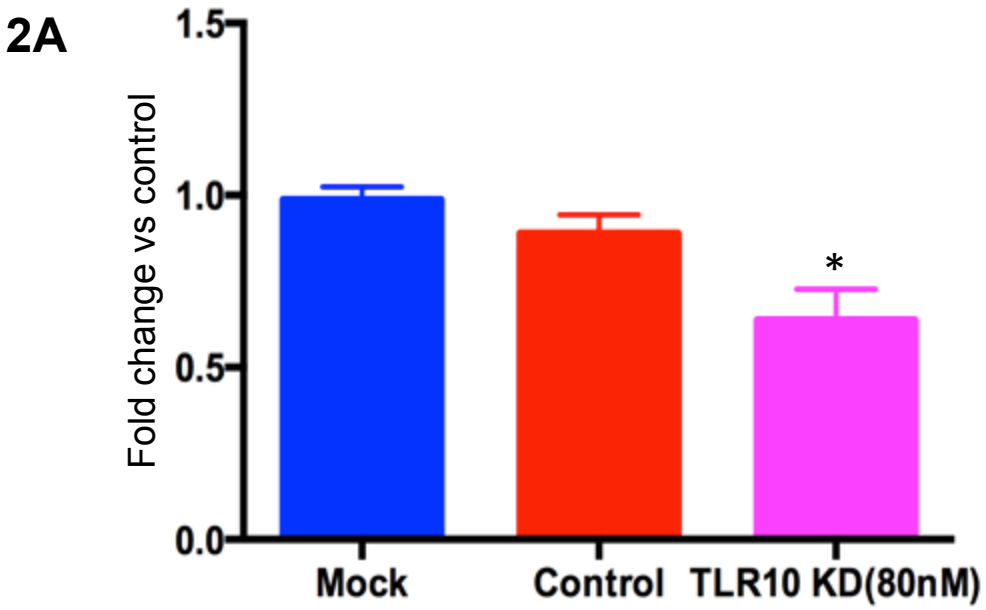


Figure 5. 1: Effect of *S. pneumoniae* in TLR10 expression in human macrophages

(A) U937 derived macrophages (1 X 10^6 cells) were infected with live invasive culture of *S. pneumoniae* (10^5 cfu, 10^6 cfu, 10^7 cfu, 10^8 cfu) for 6hr at 37°C. Total mRNA isolation was performed and is used for quantitative real time PCR. Fold change was calculated using ΔCt method. **(B)** U937 derived macrophages were (1 X 10^7 cells) infected with live invasive culture of 10^7 cfu *S. pneumoniae* for 3, 6, 12 and 24hrs. Total mRNA isolation was performed and used for quantitative real time PCR. Fold change of TLR10 mRNA was calculated using ΔΔCt method. *$p \leq 0.05$ considered as significant. **(C)** U937 derived macrophages (1 X 10^6 cells) were infected with live invasive culture of 10^7 cfu *S.*

pneumoniae for 6hr and immuno-stained for TLR10 (in green) and nucleus (in blue). Cells were imaged using Leica SP5 confocal microscopy under oil immersion (Magnification. 630). **(D)** Corrected total cell fluorescence was calculated from integrated density, area and mean fluorescence. Raw data was analyzed using t test. *$p \leq 0.05$ considered as significant. One representative of three in the above different experiments is shown.



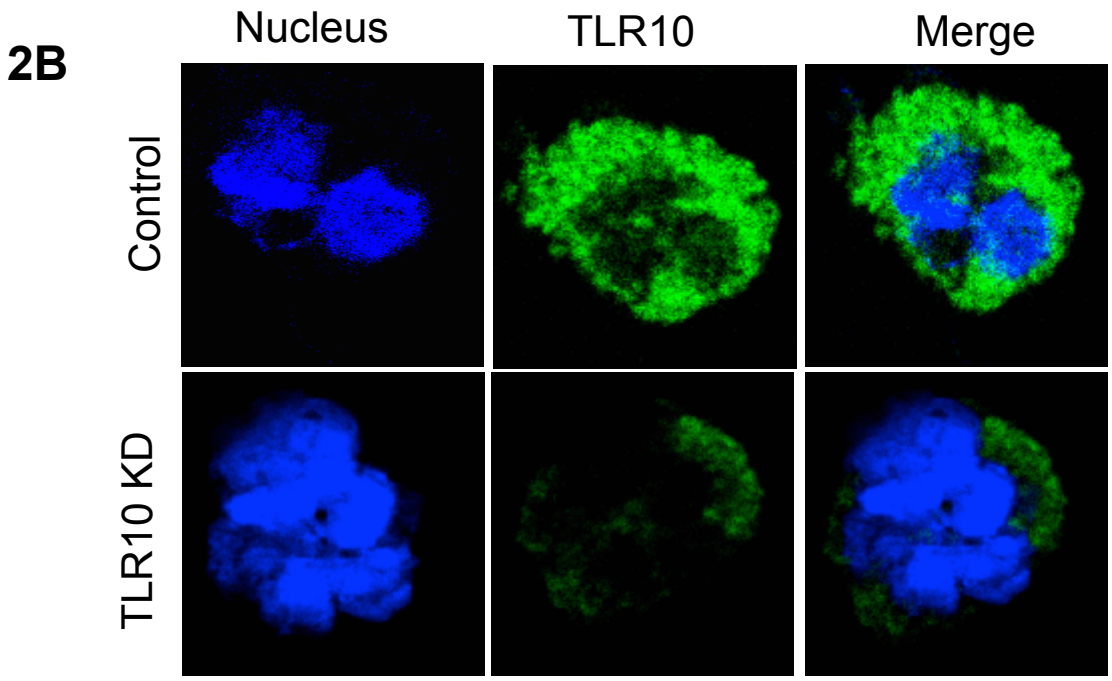


Figure 5. 2: TLR10 silencing using RNA interference

(A) U937 derived macrophages (1 X 10^6 cells) were transfected with 80nM of TLR10 siRNA (sc-40272; Santa Cruz Biotechnology) using liposome mediated transfection after

obtaining 80% confluence in culture. Total mRNA was isolated and used for quantitative real time PCR. Fold change of TLR10 mRNA was calculated using ΔΔCt method. *$p \leq 0.05$ considered as significant. (B) TLR10 silenced macrophages ($1 X 10^6$ cells) were immuno-stained for TLR10 (in green) and nucleus (in blue). Cells were imaged using Leica SP5 confocal microscopy under oil immersion (Magnification. 630). One representative of two in the above different experiments is shown.

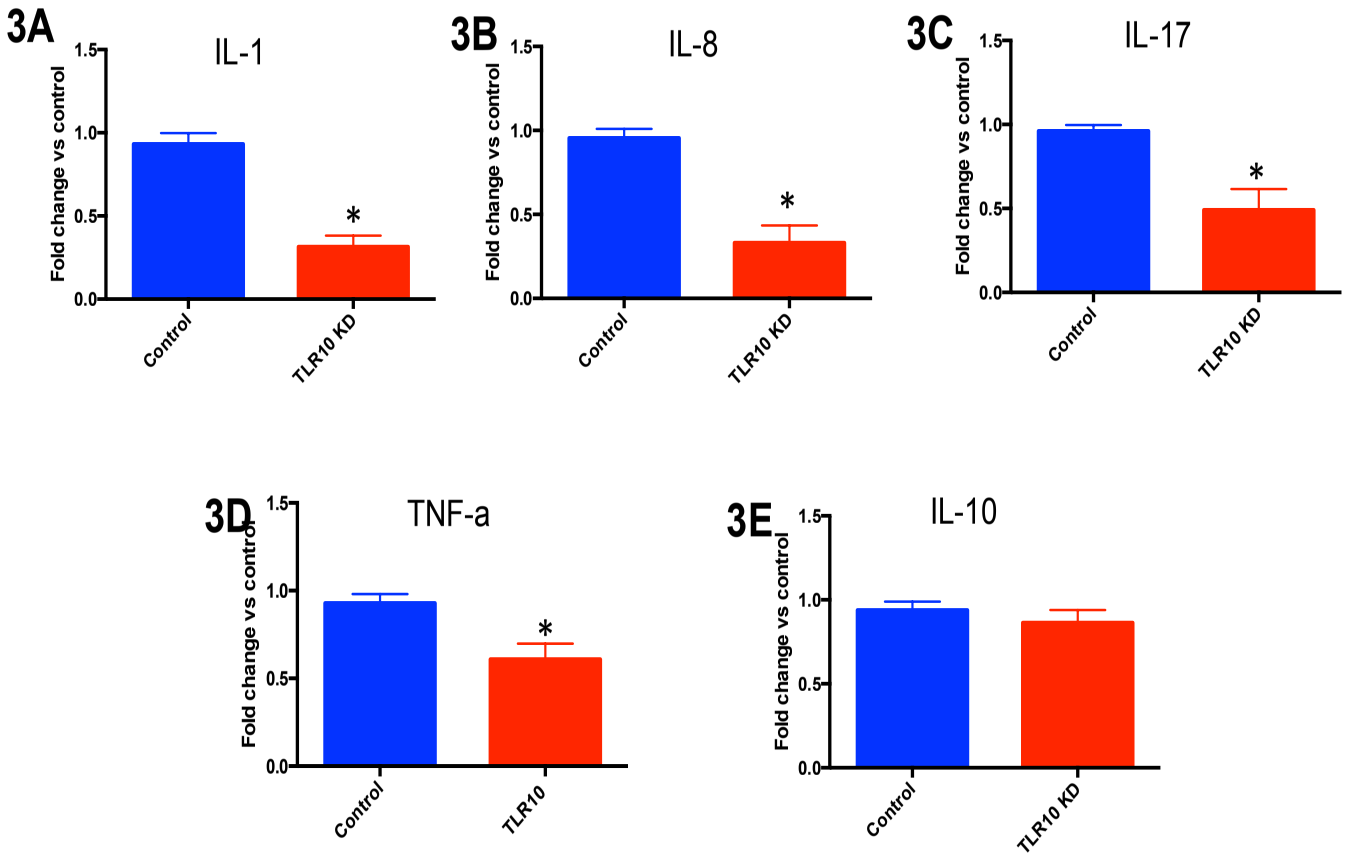

3A
IL-1
Fold change vs control
1.5
1.0
0.5
0.0
*
Control
TLR10 KD
3B
IL-8
Fold change vs control
1.5
1.0
0.5
0.0
*
Control
TLR10 KD
3C
IL-17
Fold change vs control
1.5
1.0
0.5
0.0
*
Control
TLR10 KD
3D
TNF-a
Fold change vs control
1.5
1.0
0.5
0.0
*
Control
TLR10
3E
IL-10
Fold change vs control
1.5
1.0
0.5
0.0
Control
TLR10 KD

Figure 5. 3: TLR10 silencing reduced the production of proinflammatory cytokines

U937 derived macrophages were transfected with TLR10 siRNA to silence TLR10 expression as explained. Control and TLR10 knockdown macrophages (1 X 10^6 cells) were challenged with live invasive *S. pneumoniae* (10^7 cfu, 6hr). Total mRNA isolation was performed after the incubation time and carried out quantitative real time PCR to determine the expression levels of IL-1 **(A)**, IL-8 **(B)**, IL-17 **(C)**, TNF-α **(D)** and IL-10 **(E)**. Primer sequences were listed in **Table. 1**. Fold change of TLR10 mRNA was calculated using ΔΔCt method. *$p \leq 0.05$ considered as significant. One representative of three in the above different experiments is shown.

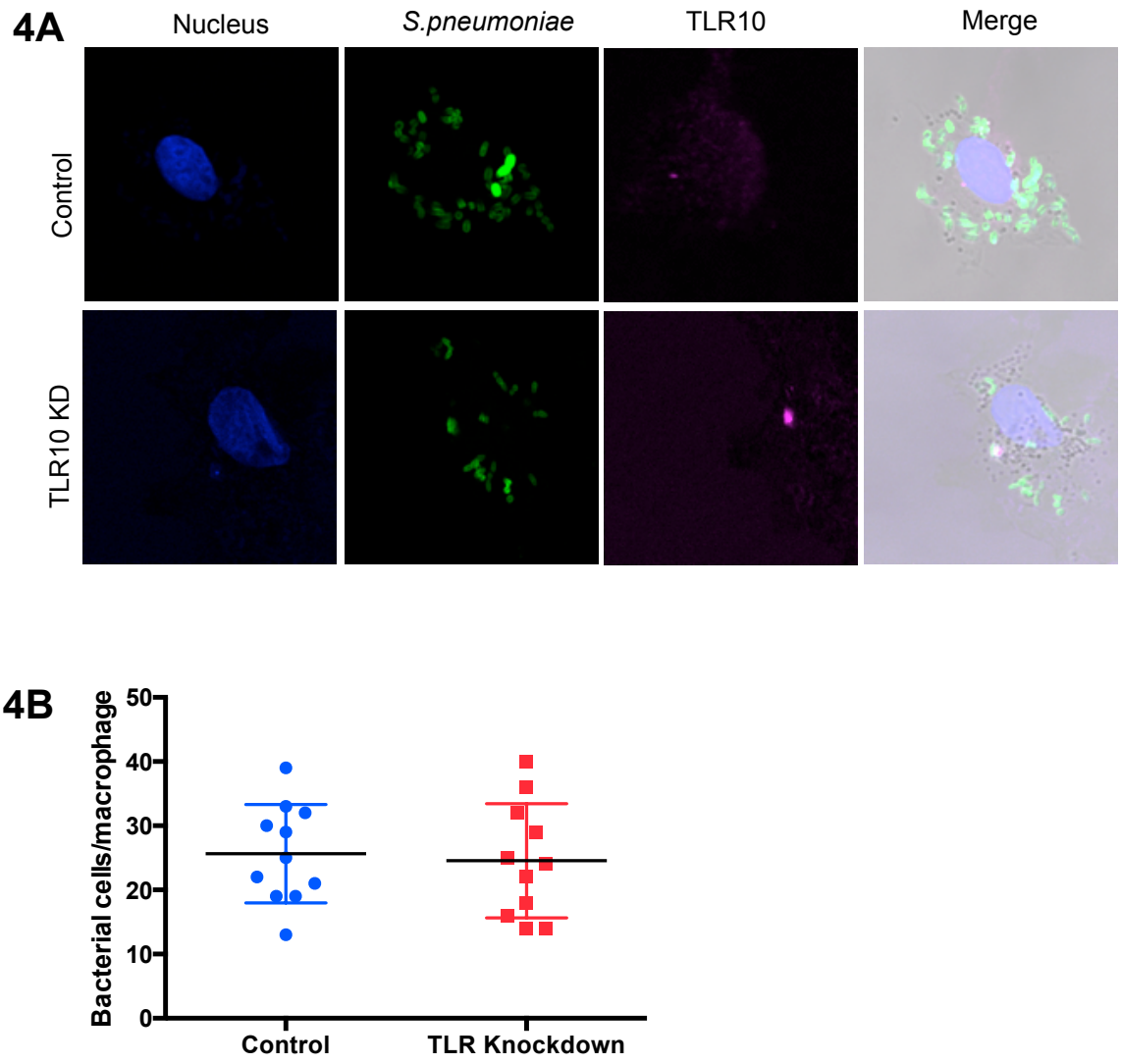


Figure 5. 4: TLR10 silencing did not affect macrophage phagocytosis

(A) Control and TLR10 silenced U937 derived macrophages (1 X 10^6 cells) were treated with fluorescent labeled live invasive culture of *S. pneumoniae* (10^7 cfu, 6hr; in green). Cells were immuno-stained for TLR10 (in pink) and nucleus (in blue). Cells were imaged using Leica SP5 confocal microscopy under oil immersion (Magnification. 630). **(B)** Number of phagocytosed bacteria was counted and analyzed using student t test. p value ≤ 0.05 considered as significant.

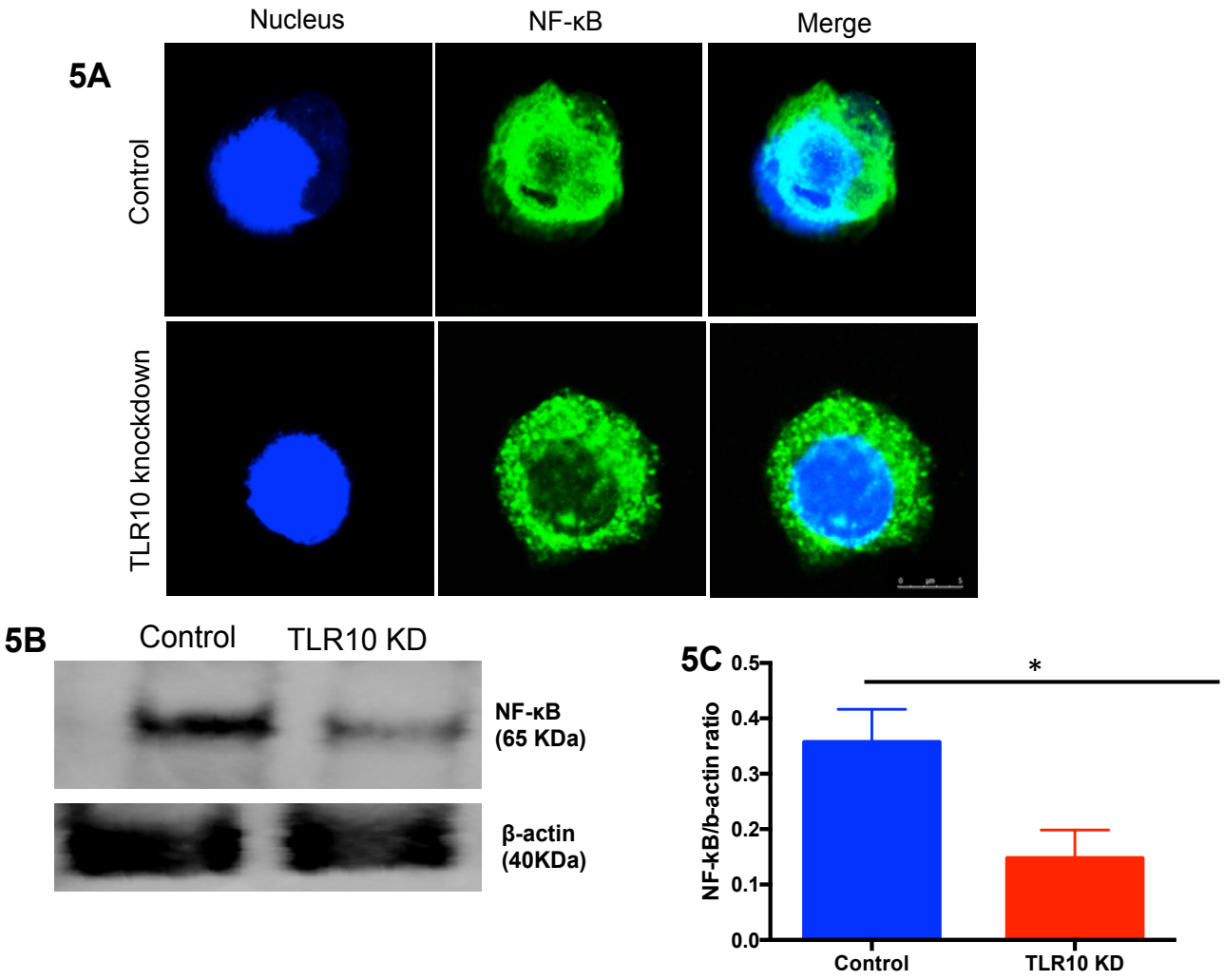


Figure 5. 5: TLR10 silencing reduced NF-κB nuclear translocation

(A) Control and TLR10 silenced U937 derived macrophages (1 X 10^6 cells) were treated with live invasive culture of *S. pneumoniae* (10^7 cfu, 6hr). Cells were immuno-stained for NF-κB (in green) and nucleus (in blue). Cells were imaged using Leica SP5 confocal microscopy under oil immersion (Magnification. 630). **(B)** Western blot of NF-κB from nuclear isolates of the macrophages (control and TLR10 knockdown) treated with 10^7 cfu *S. pneumoniae* for 6hr and **(C)** shows the densitometric quantification. One representative of three in the above different experiments is shown.

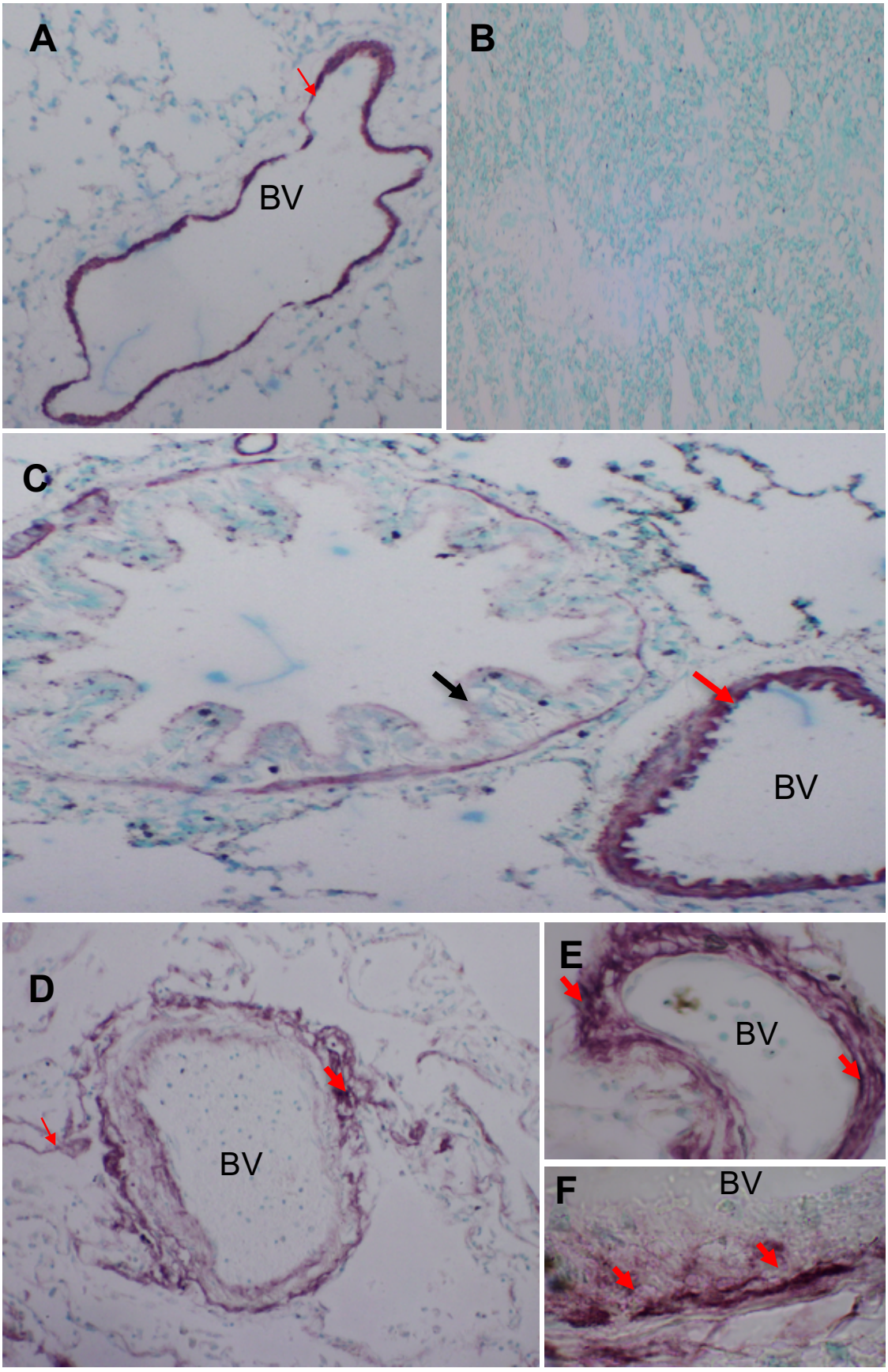

A
BV
B
C
BV
D
BV
E
BV
F
BV

Figure 5. 6: TLR10 expression in human lungs

Human lung sections were stained with vWF antibody **(A)** and show staining in endothelium (arrows) but not in bronchiolar epithelium, and isotype-matched antibody **(B)** lacks staining. Lung from a control subject **(C)** shows staining (red arrows) in endothelial area of a blood vessel (**BV**). Bronchiolar epithelium (black thick arrows) shows no staining for TLR10. The TLR10 staining is increased in a lung from an asthmatic patient **(D, E &F)** compared to the control. Magnification 100 (A-D), 400 (E) and 1000 (F).

Table 5.1: List of primers

Gene	Forward primer	Backward primer
TLR10	ACTTTGCCCACCACAATCTC	CCCAGAAAAGCCCACATTTA
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
IL-10	TGGTGAAACCCCGTCTCTAC	CTGGAGTACAGGGGCATGAT
TNF-α	ACATACTGACCCACGGCTTC	GCACTCACCTCTTCCCTCTG
IL-1β	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA

5.5 Discussion

TLRs are highly conserved innate immune receptors, which detect specific pathogen associated antigenic patterns. While TLR10 was identified a few years ago (Chuang and Ulevitch, 2001), little is known about the function and ligand of TLR10. When first identified, TLR10 mRNA expression was shown in organs such as brain, lungs, kidney, pancreas, spleen, lymph nodes and immune cells (Chuang and Ulevitch, 2001). We found that TLR10 regulates the production of IL-1, IL-8, IL-17 and TNF-α via NF-κB pathway in macrophages infected with *S. pneumoniae*. We provide the first immunohistological data that TLR10 is expressed in normal and asthmatic human lungs.

Asthmatic lungs are susceptible to viral and bacterial infections (Sutherland and Martin, 2007; Busse et al., 2010; Rosenthal et al., 2010). *S. pnuemoniae* is one of the most common bacterial infection in the lungs of humans suffering from asthma. Therefore, we examined the effects of *S. pneumoniae* infection on macrophages derived from U937 ell line. The exposure of U937 cells to *S. pneumoniae* altered the temporal and spatial expression of TLR10. TLR10 expression in the macrophage cell line was significantly increased at 10^7 and 10^8 cfu but not at lower infection dosages. Having determined dose-dependent increased in the TLR10 expression, we further found that increase in expression was also time-dependent with maximal expression observed at 6hr. Interestingly, TLR10 mRNA was not detected at 12hr post-infection but was observed at 24hr. The mRNA data were supported through TLR10 protein immunofluorescence experiments that provided evidence of increased TLR10 expression in the cytoplasm of macrophages. Taken together these are the first data to show that *S. pneumoniae* infection in macrophages upregulates TLR10 expression in a dose and time dependent manner.

Bacterial infections activate immune cells such as macrophages leading to production of variety of inflammatory mediators (O'Riordan et al., 2002; Torraca et al., 2014; Wegiel et al., 2014). We used a panel of inflammatory mediators as a biological readout of the role of TLR10 in *S. pneumoniae* infection through knocking down TLR10 in the macrophage cells. Through the use of siRNA, the TLR10 mRNA and protein expression was significantly reduced. We used this system to assess the effect of TLR10 on the production of selected cytokines. We selected IL-1β, IL-8, IL-17 and TNF-α for their roles as pro-inflammatory cytokines in bacterial infections and IL10 as a regulatory and anti-inflammatory cytokine (Cavaillon, 1994; Stow et al., 2009; Varin and Gordon, 2009; Scull et al., 2010; Lacy and Stow, 2011). The production of IL-1β, IL-8, IL-17 and TNF-α mRNA but not IL10 mRNA was significantly reduced in the macrophages with TLR10 knockdown to suggest its role in production of these inflammatory mediators in macrophages infected with *S. pneumoniae*. Host response against *S. pneumoniae* infection includes the activation of humoral and cell mediated immunity along with changes in expression levels of cytokines and chemokines (Wilson et al., 2014). Changes in cytokine mRNA levels in *S. pneumoniae* infected TLR10 knockdown macrophages, suggesting TLR10 might involve in the immune sensing of gram-positive bacterial infections. Regan et al showed the proinflammatory role of TLR10 in *L. monocytogenes* infection in THP-1 cells (Regan et al., 2013). The data show a novel role for TLR10 in the production of inflammatory mediators in macrophages infected with *S. pneumoniae*.

We further explored the mechanisms through which TLR10 may be regulating the cytokine production. First, we examined whether TLR10 affects macrophages ability for

phagocytosis. The experiment using *S. pneumoniae* labeled with fluorescent markers showed that TLR10 knockdown did not affect phagocytosis. This is in contrast to the role that TLR4, which has a positive effect on macrophage phagocytosis *E. coli* by interperitoneal macrophages (Anand et al., 2007). Among the TLRs, TLR9 being the strongest and TLR3 being the weakest inducer of macrophage phagocytosis through regulating interleukin-1 receptor–associated kinase-4 and p38 signalling (Doyle et al., 2004). Our data shows TLR10 doesn't regulate phagocytosis of *S. pneumoniae* by macrophages. After this we examined whether TLR10 affects transcription factor NF-κB, and found that TLR10 knockdown leads to a reduction in the expression of NF-κB and its reduced localization into the nucleus of infected macrophages. Previously, NF-κB mobilization by hypoxia has been shown to upregulate TLR10 mRNA (Kim et al., 2010). Our data show that TLR10 regulates production of inflammatory cytokines through activating the nuclear translocation of NF-kB but through affect on the phagocytosis of bacteria.

The finding of immunomodulatory roles of TLR10 has important consequences. First of all, this study elucidates a biological function for TLR10 in macrophages' response against *S. pneumoniae*. The data assume significance because TLR10 is still one of the least studied TLR largely due to lack of knowledge on the identity of its ligand and lack of functional TLR10 in mice. The ability of bacteria to alter TLR10 expression and the regulation of cytokine production by TLR10 in infected macrophages will create opportunities to better understand the mechanisms of bacterial disease such as that caused by *S. pneumoniae*. The production of pro-inflammatory cytokines but not anti-inflammatory IL10 by *S. pneumoniae* via TLR10 pathway could be an important

component of host's immune response, which these bacteria may subvert. Such insights may be relevant for the development of novel treatment strategies based on modulation of the function of TLR10.

CHAPTER 6: GENERAL DISCUSSION

Primary aims of this project were to investigate the expression and function of TLR10 in immune cells, and to delineate expression of TLR10 in lung of humans as well as in commercially important domestic animal species. Since TLRs are the guardians of innate immune system, I examined the role of TLR10 in neutrophil chemotaxis and innate immune response towards *S. pneumoniae* infection. We report previously unknown roles of TLR10 in neutrophil chemotaxis and responses of macrophages towards *S. pneumoniae*, which will enhance our understanding of fundamental biology of TLR10 and mechanisms of inflammatory diseases.

I have characterized the expression of TLR10 in chicken, cattle, pig, dog, rats, and human lungs. Prior to our study, there were no data available on the expression of TLR10 in lungs of these species. However, the expression kinetics of human TLR10 in intestine, tonsils and in liver has been reported recently (Chuang and Ulevitch, 2001; Bourke et al., 2003; Regan et al., 2013). First, I performed homology analysis and protein sequence alignment of the peptide used to produce a commercially available anti-human TLR10 polyclonal antibody against the TLR10 peptide sequence of chicken, cattle, pig, rat, and dog. This was needed because of a lack of a species-specific commercial antibody for use in these species. Because the spatial cell-specific expression is highly informative in understanding the role of molecules such as TLR10, I used *in situ* light and electron microscopic immunochemistry. The expression of TLR10 was located in the lungs of all the species, and the electron microscopy showed TLR10 in the cytoplasm, plasma membrane and nucleus of lung cells. TLR10 expression was also observed in pulmonary intravascular macrophages, which are a unique highly phagocytic population of

macrophages present in species such as cattle, sheep, goat, pigs, and horse and phagocytose blood-borne particles (Winkler, 1988; Chitko-McKown et al., 1991; Singh, 2004; Schneberger et al., 2012). I found evidence of altered expression of TLR10 in inflamed lungs from pig, cattle and chicken. The alterations in cell-specific *in situ* TLR10 data in inflamed lungs compared to the normal suggest a potential regulatory effect of the microbes on the expression of TLR10. The data on the expression of TLR10 does set the stage to explore its specific roles in lung biology

The immune cells such as neutrophils are the first responders in acute inflammation including that in the lungs (Snyder et al., 1991; Pasparakis and Vandenabeele, 2015). Because the identity of ligand for TLR10 is unknown, I used *E. coli* LPS as an agonist to stimulate neutrophils. Because the *in situ* data from multiple species showed TLR10 in neutrophils located in the lungs, I examined the expression of TLR10 in normal neutrophils and those treated with *E. coli* LPS. While the LPS treatment did not change the amount of TLR10 in the neutrophils, there were alterations in spatial expression. Following this, I examined the human peripheral blood neutrophils. As the first step to understand TLR10 biology, I used multiple methods to examine the TLR10 expression in detail and the mechanisms that regulate its expression in neutrophils. The activated neutrophils showed that lipid-raft mediated endocytosis of TLR10 altered its localization on the plasma membrane. There are many other instances where lipid-rafts have been shown to influence the dynamics of molecules including TLRs located in the plasma membrane of various cells (Szabo et al., 2007; Daniel and Kai, 2010; Zhu et al., 2010). An interesting aspect of the data is that TLR4-mediated production of ROS and nuclear translocation of NF-κB are the key regulators of

expression of TLR10. NF-κB activation is reported to have effect on the changes in expression of TLR2, 4 and 9 mRNA in human dendritic cells (An et al., 2002; Kawai and Akira, 2007).

During a curiosity-driven live cell imaging experiment, which was prompted by immuno-electron microscopic localization of TLR10 on the leading edge of neutrophils, I observed dynamic localization of TLR10 into the pseudopods of activated neutrophils. It is well known that neutrophils upon activation migrate towards the site of inflammation or infection as one of their primal function (Zachariae, 1993; Cicchetti et al., 2002). There are few existing data on the role of TLRs, especially TLR4, in neutrophil chemotaxis. Many of these data on the role of TLRs in neutrophil chemotaxis are contradictory (Fan and Malik, 2003a; Hayashi et al., 2003; Khan et al., 2005; Sabroe et al., 2005; Aomatsu et al., 2008b; Alves-Filho et al., 2009; Yi et al., 2012b). This experiment prompted further experiments including the use of HL-60 cells to examine the role of TLR10 in chemotaxis. Using siRNA to knockdown TLR10, I did observe that TLR10 is required for fMLP-directed migration of neutrophils. The TLR10 knockdown reduced the number of pseudopods on activated neutrophils. My efforts to establish a mechanism for the role TLR10 in the formation of pseudopods via regulation of actin-nucleation proteins were not conclusive. Therefore, further experiments are needed to address the mechanisms through which TLR10 regulates the migration of neutrophils. This however does not affect the novelty of the observations that TLR10 plays a role in the migration of neutrophils because it advances our understanding of mechanisms of migration of neutrophils, which are incompletely understood.

Asthmatic lungs are susceptible to viral and bacterial infections and *S. pneumoniae* is one of the most common bacterial infections in the lungs of humans suffering from asthma (Sutherland and Martin, 2007; Busse et al., 2010; Rosenthal et al., 2010). Macrophages are credited with regulation of lung inflammation in variety of conditions including asthma (Wojcik et al., 2008; Herold et al., 2011; Hiraiwa and van Eeden, 2013). Because I reported the expression of TLR10 in normal and asthmatic lungs, I proceeded to examine the question whether TLR10 has a role in the responses of U937-derived macrophages against *S. pneumoniae* infection. Again using the siRNA technology, I found that reduced expression of TLR10 significantly reduced the production of IL-1β, IL-8, IL-17 and TNF-α mRNA but not IL10 mRNA in macrophages infected with *S. pneumoniae.* However, the phagocytosis of the bacteria was not affected. This is in contrast the data that TLR3 and 4 regulate phagocytosis by macrophages (Anand et al., 2007). These are intriguing data because bacteria may down-regulate TLR10 expression to reduce production of cytokines and potency of the inflammatory response to avoid their elimination. I believe further experiments are needed to find out whether TLR10 has a function in the degradation of bacteria by the macrophages.

Taken together these data show that there is a homologous and constitutive expression of TLR10 across different species. Further, when TLR10 was silenced in neutrophils and macrophages exposed to LPS and *S. pneumoniae*, respectively, there were significant alteration in their chemotactic and immune responses. The finding of potential functional roles of TLR10 has significant consequences. Importantly, this work explains the possible biological function for TLR10 in response to bacterial LPS and *S. pneumoniae*, contributes to our understanding of TLR biology and better insight into

pathology of bacterial infections. Such insights may be relevant for the development of novel treatment strategies based on modulation of the function of TLR10.

CHAPTER 7: LIMITATIONS OF THE STUDY

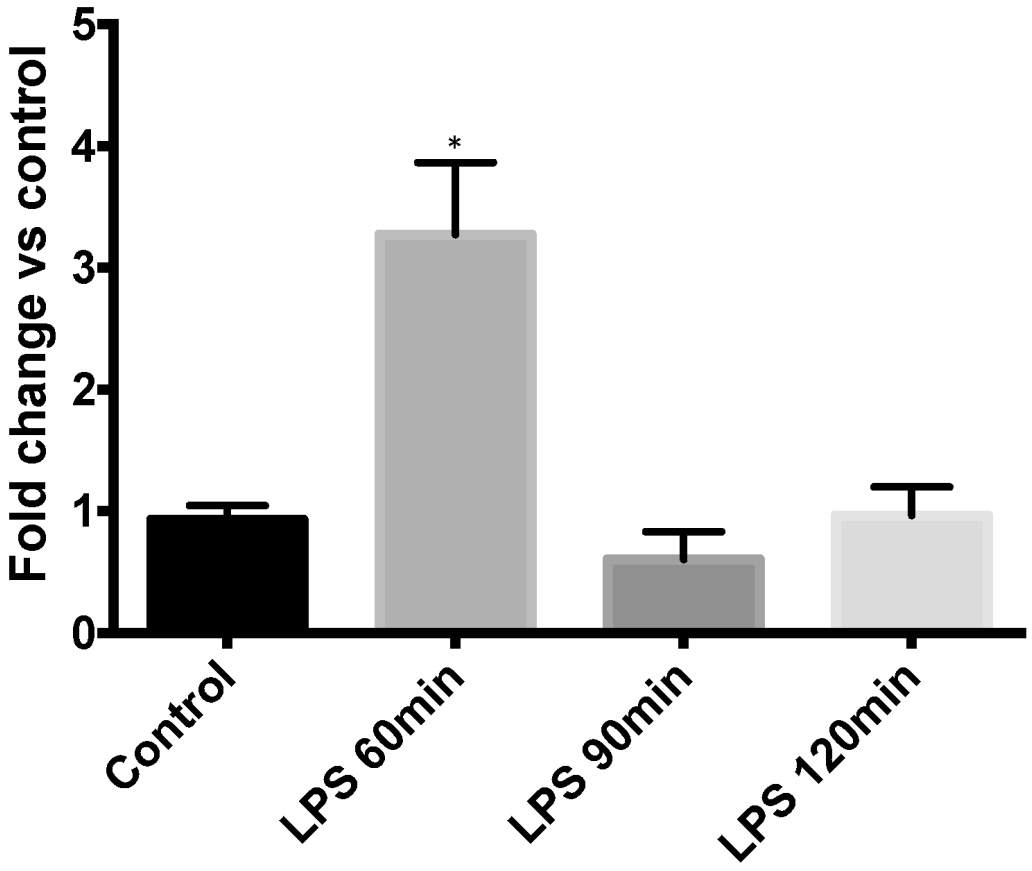
In spite of the fact that the data from my study furthers our understanding on functional role of human TLR10, there are some limitations as well. This study couldn't provide the conclusive evidence of mechanisms through which TLR10 alters neutrophil chemotaxis. The study does not provide protein data to support the transcriptional level changes in pro-inflammatory mediators in macrophages infected with *S. pneumoniae*. The work on domestic animal species also utilized lungs from various species that were infected by bacteria or viruses or injected with endotoxin thus leading to difficulty in comparing data across species with lung inflammation.

CHAPTER 8: FUTURE DIRECTIONS

Future studies are required to explain the molecular mechanisms through which TLR10 regulates neutrophil chemotaxis. Since TLR10 is not functional in mice, there also is a need to develop a new model system to study the functional aspects of TLR10 in innate immunity. I have only examined isolated primary neutrophils and U937 derived macrophages for TLR10 expression and dynamics. Hence these studies should be performed in a validated animal model in order to exploit the TLR10 as a potential drug target.

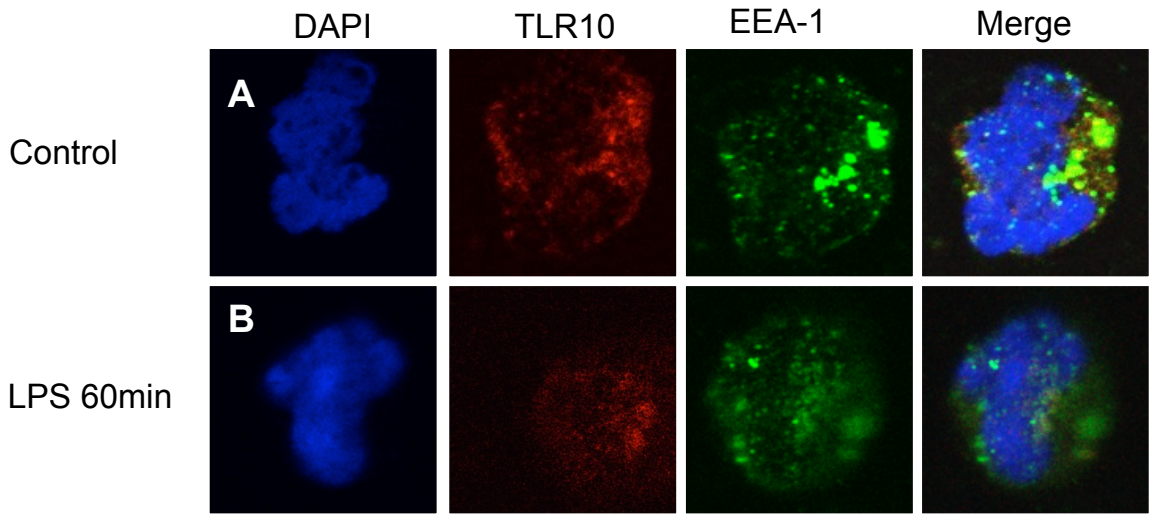
APPENDIX

Supplementary figures



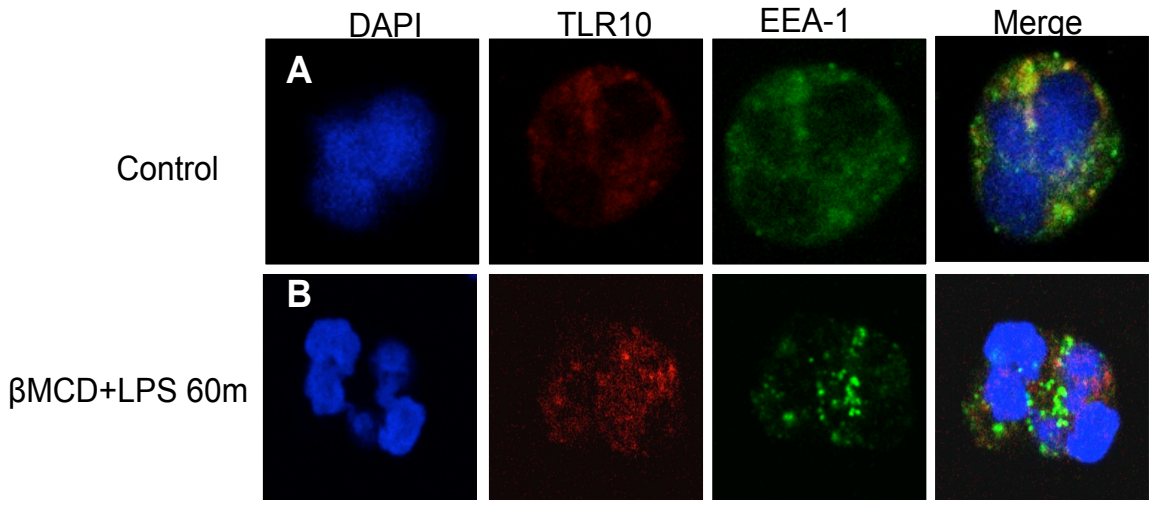
Supplementary Figure 4.1: LPS (60min) challenge upregulated TLR10 mRNA

Human neutrophils were (1 X 10^7 cells) treated with LPS (1μg/ml) for 60, 90 and 120min. Total mRNA isolation was performed and used for quantitative real time PCR. Fold change of TLR10 mRNA was calculated using ΔΔCt method. *$p \leq 0.05$ vs control, considered as significant.



Supplementary Figure 4.2: Low temperature disruption of endocytic pathway inhibited TLR10 internalization

(A, B) Human neutrophils (1 X 10^6) adhered to FBS coated coverslips; low temperature disruptions of endocytic pathway were performed and were activated by LPS (1μg/ml). Merged panel shows TLR10 (red), nucleus (blue) and early endosomal antigen, EEA1 (green). Note the lack of colocalization in panel. B.



Supplementary Figure 4.3: Lipid raft disruption inhibited TLR10 endocytosis

(A, B) Human neutrophils (1 X 106) adhered to FBS coated coverslips; low temperature disruptions of endocytic pathway were performed and were activated by LPS (1μg/ml). Merged panel shows TLR10 (red), nucleus (blue) and early endosomal antigen, EEA1 (green). Note the lack of colocalization in panel. B

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Abad C, González-Escribano MF, Diaz-Gallo LM, Lucena-Soto JM, Márquez JL, Leo E, Crivell C, Gómez-García M, Martín J, Núñez-Roldán A, García-Lozano JR. 2011. Association of Toll-like receptor 10 and susceptibility to Crohn's disease independent of NOD2. Genes and Immunity.

Aboussahoud W, Aflatoonian R, Bruce C, Elliott S, Ward J, Newton S, Hombach-Klonisch S, Klonisch T, Fazeli A. 2010. Expression and function of Toll-like receptors in human endometrial epithelial cell lines. J Reprod Immunol 84:41-51.

Akira S. 2003. Toll-like receptor signaling. J Biol Chem 278:38105-38108.

Akira S, Takeda K. 2004. Toll-like receptor signalling. Nature reviews Immunology 4:499-511.

Akira S, Takeda K, Kaisho T. 2001. Toll like receptors: Critical proteins linking innate and acquired immunity. Nature Reviews 2:675-680.

Akira S, Uematsu S, Takeuchi O. 2006. Pathogen recognition and innate immunity. Cell 124:783-801.

Albrecht I, Tapmeier T, Zimmermann S, Frey M, Heeg K, Dalpke A. 2004. Toll-like receptors differentially induce nucleosome remodelling at the IL-12p40 promoter. EMBO reports 5:172-177.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. 2001. Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3. Nature 41:732-738.

Alves-Filho J, Freitas A, Souto F, Spiller F, Paula-Neto H, Silva J, Gazzinelli R, Teixeira M, Ferreira S, Cunha F. 2009. Regulation of chemokine receptor by Toll-like

receptor 2 is critical to neutrophil migration and resistance to polymicrobial sepsis. PNAS 106:4018-4023.

Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. 2012. Neutrophil function: from mechanisms to disease. Annu Rev Immunol 30:459-489.

An H, Yu Y, Zhang M, Xu H, Qi R, Yan X, Liu S, Wang W, Guo Z, Guo J, Qin Z, Cao X. 2002. Involvement of ERK, p38 and NF-kB signal transduction in regulation of TLR2, TLR4 and TLR9 gene expression induced by lipopolysaccharide in mouse dendritic cells. Immunology 106:38-38.

Anand RJ, Kohler JW, Cavallo JA, Li J, Dubowski T, Hackam DJ. 2007. Toll-like receptor 4 plays a role in macrophage phagocytosis during peritoneal sepsis. Journal of Pediatric Surgery 42:927-933.

Anderson K, Bokla L, Nusslein-Volhard C. 1985. Establishment of dorsal-ventral polarity in the Drosophila embryo: The induction of polarity by the toll gene Ppoduct. Cell 42:791-798.

Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, Kubes P. 2003. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. The Journal of clinical investigation 111:1011-1020.

Aomatsu K, Kato T, Fujita H, Hato F, Oshitani N, Kamata N, Tamura T, Arakawa T, Kitagawa S. 2008a. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. Immunology 123:171-180.

Aomatsu K, Kato T, Fujita H, Hato F, Oshitani N, Kamata N, Tamura T, Arakawa T, S SK. 2008b. Toll-like receptor agonists stimulate human neutrophil migration via activation of mitogen-activated protein kinases. Immunology 123:171-180.

Arraes SM, Freitas MS, da Silva SV, de Paula Neto HA, Alves-Filho JC, Auxiliadora Martins M, Basile-Filho A, Tavares-Murta BM, Barja-Fidalgo C, Cunha FQ. 2006. Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation. Blood 108:2906-2913.

Aschenbroich S, Nemeth N, Rech R, Briggs R, Sanchez S, Brown C. 2013. Mannheimia haemolytica A1-induced Fibrinosuppurative Meningoencephalitis in a Naturally-infected Holstein-Friesian Calf. Journal of comparative pathology:161-171.

Asehnoune K, Strassheim D, Mitra S, Kim JY, Abraham E. 2004. Involvement of Reactive Oxygen Species in Toll-Like Receptor 4-Dependent Activation of NF-kB. J Immunol 172:2522-2529.

Asthma.ca. 2011. Asthma facts and statistics In: Asthma facts and statistics Asthma.ca: ASC.

Bafica A, Scanga CA, Feng CG, Leifer C, Cheever A, Sher A. 2005. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. The Journal of experimental medicine 202:1715-1724.

Balachandran Y, Knaus S, Caldwell S, Singh B. 2015. Toll-like receptor 10 expression in chicken, cattle, pig, dog, and rat lungs. Vet Immunol Immunpathol 168:184-192.

Balhara J, Gounni AS. 2012. The alveolar macrophages in asthma: a double-edged sword. Mucosal immunology 5:605-609.

Barbalat R, Lau L, Locksley RM, Barton GM. 2009. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol 10:1200-1207.

Barbara Koller MK, Phillip Latzin, Amit Gaggar, Marcus Schreiner, Sherkin Takyar, Michael Kormann, Michael Kabesch, Dirk Roos, Matthias Griese and Dominik Hartl. 2008. TLR Expression on Neutrophils at the Pulmonary Site of Infection: TLR1/TLR2-Mediated Up-Regulation of TLR5 Expression in Cystic Fibrosis Lung Disease. J Immunol 181:2753-2763.

Barnes PJ. 2004. Mediators of chronic obstructive pulmonary disease. Pharmacological reviews 56:515-548.

Barton GM, Kagan JC, Medzhitov R. 2005. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nature Immunology 7:49-56.

Basu S, Fenton MJ. 2004. Toll-like receptors: function and roles in lung disease. Am J Physiol Lung Cell Mol Physiol 286:L887-892.

Beutler B. 2009. TLRs and Inate Immunity. Blood 113:1399 - 1407.

Bianchi S, Dockrell D, Renshaw S, Sabroe I, Whyte M. 2006. Granulocyte apoptosis in the pathogenesis and resolution of lung disease. Clin Sci 110:293-304.

Bogaert D, de Groot R, Hermans PWM. 2009. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. The Lancet Infectious Diseases 4:144-154.

Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A. 2003. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 102:956-963.

Boyd AR, Shivshankar P, Jiang S, Berton MT, Orihuela CJ. 2012. Age-related defects in TLR2 signaling diminish the cytokine response by alveolar macrophages during murine pneumococcal pneumonia. Experimental gerontology 47:507-518.

Branzk N, Papayannopoulos V. 2013. Molecular mechanisms regulating NETosis in infection and disease. Seminars in immunopathology 35:513-530.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A. 2004. Neutrophil Extracellular Traps Kill Bacteria. Science 303:1532-1535.

Brownlie R, Allan B. 2010. Avian toll-like receptors. Cell and Tissue Research 343:121-130.

Burns A, Smith C, Walker D. 2003. Unique structural features that influence neutrophil emigration into the lung. Physiol Rev 83:309-336.

Busse WW, Lemanske RF, Gern JE. 2010. Role of viral respiratory infections in asthma and asthma exacerbations. The Lancet 376:826-834.

Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller-Graziano C, Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG, Lowry SF, Inflamm, Host Response to Injury Large Scale Collab. Res P. 2005. A network-based analysis of systemic inflammation in humans. Nature 437:1032-1037.

Cassol E, Cassetta L, Rizzi C, Alfano M, Poli G. 2009. M1 and M2a polarization of human monocyte-derived macrophages inhibits HIV-1 replication by distinct mechanisms. J Immunol 182:6237-6246.

Cavaillon J. 1994. Cytokines and macrophages Biomed Pharmacother 48:445-453.

Chen YC, Giovannucci E, Kraft P, Lazarus R, Hunter DJ. 2007. Association between Toll-like receptor gene cluster (TLR6, TLR1, and TLR10) and prostate cancer. Cancer Epidemiol Biomarkers Prev 16:1982-1989.

Chiavolini D, Pozzi G, Ricci S. 2008. Animal models of Streptococcus pneumoniae disease. Clinical microbiology reviews 21:666-685.

Chiers K, Donne E, Overbeke IV, Ducatelle R, Haesebrouck F. 2002. Evaluation of serology, bacteriological isolation and polymerase chain reaction for the detection of pigs carrying Actinobacillus pleuropneumoniae in the upper respiratory tract after experimental infection. Vet Microbiol 88:385-392.

Chiron D, Bekeredjian-Ding I, Pellat-Deceunynck C, Bataille R, Jego G. 2008. Toll-like receptors: lessons to learn from normal and malignant human B cells. Blood 112:2205-2213.

Chitko-McKown CG, Chapes SK, Brown RE, Phillips RM, McKown RD, Blecha F. 1991. Porcine Alveolar and Pulmonary Intravascular Macrophages: Comparison of Immune Functions. J leuko biol 50:364-372.

Chuang T, Ulevitch RJ. 2001. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta 1518:157-161.

Cicchetti G, Allen PG, Glogauer M. 2002. Chemotactic Signaling Pathways in Neutrophils: from Receptor to Actin Assembly. Critical Reviews in Oral Biology & Medicine 13:220-228.

Confer AW. 1993. Immunogens of Pasteurella. Vet Microbiol 37:353-368.

Covarrubias A, Byles V, Horng T. 2013. ROS sets the stage for macrophage differentiation. Cell Res 23:984-985.

Daniel L, Kai S. 2010. Lipid rafts as a membrane- organizing principle. Science 327:46-50.

de Vos AF, Dessing MC, Lammers AJ, de Porto AP, Florquin S, de Boer OJ, de Beer R, Terpstra S, Bootsma HJ, Hermans PW, van 't Veer C, van der Poll T. 2015. The polysaccharide capsule of Streptococcus pneumonia partially impedes MyD88-mediated immunity during pneumonia in mice. PLoS One 10:e0118181.

DeAlessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, Pipeling MR, Brower RG, Tuder RM, McDyer oF, King LS. 2009. CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. The Journal of clinical investigation 119:2898–2913.

Deng M, Scott MJ, Loughran P, Gibson G, Sodhi C, Watkins S, Hackam D, Billiar TR. 2013. Lipopolysaccharide clearance, bacterial clearance, and systemic inflammatory responses are regulated by cell type-specific functions of TLR4 during sepsis. J Immunol 190:5152-5160.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303:1529-1531.

Donahoe M. 2011. Acute respiratory distress syndrome: A clinical review. Pulmonary circulation 1:192-211.

Douda DN, Yip L, Khan MA, Grasemann H, Palaniyar N. 2014. Akt is essential to induce NADPH-dependent NETosis and to switch the neutrophil death to apoptosis. Blood 123:597-600.

Doyle SE, O'Connell RM, Miranda GA, Vaidya SA, Chow EK, Liu PT, Suzuki S, Suzuki N, Modlin RL, Yeh WC, Lane TF, Cheng G. 2004. Toll-like receptors induce a phagocytic gene program through p38. The Journal of experimental medicine 199:81-90.

Dubourdeau M, Athman R, Balloy V, Huerre M, Chignard M, Philpott DJ, Latgé J-P, Ibrahim-Granet Om. 2006. Aspergillus fumigatus Induces Innate Immune Responses in Alveolar Macrophages through the MAPK Pathway Independently of TLR2 and TLR4. J Immunol 177:3994-4001.

Etem EO, Elyas H, Ozgocmen S, Yıldırım A, Godekmerdan A. 2011. The investigation of toll-like receptor 3, 9 and 10 gene polymorphisms in Turkish rheumatoid arthritis patients. Rheumatol Int 31:1369-1374.

Ewers C, Lübke-Becker A, Wieler L. 2004. Mannheimia haemolytica and the pathogenesis of enzootic bronchopneumonia. Berl Munch Tierarztl Wochenschr 117:97-115.

Fan J. 2009. TLR Cross-Talk Mechanism of Hemorrhagic Shock-Primed Pulmonary Neutrophil Infiltration. The Open Critical Care Medicine Journal 2:1-8.

Fan J, Frey RS, Malik AB. 2003. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. The Journal of clinical investigation 112:1234-1243.

Fan J, Malik A. 2003a. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. Nat Med 9:315-321.

Fan J, Malik AB. 2003b. Toll-like receptor-4 (TLR4) signaling augments chemokine-induced neutrophil migration by modulating cell surface expression of chemokine receptors. Nat Med 9:315-321.

Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, Buwitt-Beckmann U, Roschmann K, Jung G, Wiesmuller KH, Ulmer AJ. 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. Journal of Leukocyte Biology 83:692-701.

Fecho K, Cohen PL. 1998. Fas ligand (gld)- and Fas (lpr)-deficient mice do not show alterations in the extravasation or apoptosis of inflammatory neutrophils. J Leukoc Biol 64:373–383.

Fujishima S. 2011. Epithelial cell restoration and regeneration in inflammatory lung diseases. Inflammation and Regeneration 31:290-296.

Futosi K, Fodor S, Mocsai A. 2013. Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol 17:638-650.

Ghosh TK, Mickelson DJ, Solberg JC, Lipson KE, Inglefield JR, Alkan SS. 2007. TLR-TLR cross talk in human PBMC resulting in synergistic and antagonistic

regulation of type-1 and 2 interferons, IL-12 and TNF-alpha. Int Immunopharmacol 7:1111-1121.

Gon S, Gatanaga T, Sendo F. 1996. Involvement of two types of TNF receptor in TNF-alpha induced neutrophil apoptosis. Microb Immunol 40:463-465.

Goodnow R. 1980. Biology of Bordetella bronchiseptica. Microbiol Rev 80:722-738.

Gordon SB, Irving GRB, Lawson RA, Lee ME, Read RC. 2000. Intracellular trafficking and killing of Streptococcus pneumoniae by human alveolar macrophages are influenced by opsonins. Infect Immun 68:2286–2293.

Grommes J, Soehnlein O. 2010. Contribution of Neutrophils to Acute Lung Injury. Mol Med 17:293-307.

Guan Y, Ranoa DR, Jiang S, Mutha SK, Li X, Baudry J, Tapping RI. 2010. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. J Immunol 184:5094-5103.

Guirado M, Gil H, Saenz-Lopez P, Reinboth J, Garrido F, Cozar JM, Ruiz-Cabello F, Carretero R. 2012. Association between C13ORF31, NOD2, RIPK2 and TLR10 polymorphisms and urothelial bladder cancer. Hum Immunol 73:668-672.

Halilovic I, Wu J, Alexander M, Lin F. 2015. Neutrophil migration under spatially-varying chemoattractant gradient profiles. Biomedical microdevices 17:9963.

Halliwell B, Zhao K, Whiteman M. 1999. Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. Free Radic Res 31:651-669.

Han C, Jin J, Xu S, Liu H, Li N, Cao X. 2010. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. Nature Immunology 11:734-742.

Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, Guiet C, Brière F, Vlach J, Lebecque S, Trinchieri G, Bates EE. 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol 174:2942-2950.

Hashimoto C, Hudson M, Anderson K. 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52:269-279.

Hayashi F, Means TK, Luster AD. 2003. Toll-like receptors stimulate human neutrophil function. Blood 102:2660-2669.

Hayashi F, Smith KD, Ozinsky d, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. 2001. The innate immune response to bacterial ŧagellin is mediated by Toll-like receptor 5. Nature 410:1099-1102.

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303:1526-1529.

Herold S, Mayer K, Lohmeyer J. 2011. Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. Frontiers in immunology 2:65.

Hertzog PJ, O'Neill LA, Hamilton JA. 2003. The interferon in TLR signaling: more than just antiviral. Trends in Immunology 24:534-539.

Hiraiwa K, van Eeden SF. 2013. Contribution of lung macrophages to the inflammatory responses induced by exposure to air pollutants. Mediators of inflammation 2013:619523.

Hong-Geller E, Chaudhary A, Lauer S. 2008. Targeting Toll-Like Receptor Signaling Pathways for Design of Novel Immune

Therapeutics. Current Drug Discovery Technologies 5:29-38.

Huang Y, Temperley ND, Ren L, Smith J, Li N, Burt DW. 2011. Molecular evolution of the vertebrate TLR1 gene family--a complex history of gene duplication, gene conversion, positive selection and co-evolution. BMC Evol Biol 11:149.

Ian R. Rifkin EAL, Liliana Busconi, Gregory Viglianti & Ann Marshak-Rothstein. 2005. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. Immunological Reviews 204:27-42.

Imai Y, Kuba K, Rao S, Huan Y, Guo F, Guan B. 2005. Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature 436:112-116.

Inoue H, Shimada A, Kaewamatawong T, Naota M, Morita T, Ohta Y, Inoue K, Takano H. 2009. Ultrastructural changes of the air-blood barrier in mice after intratracheal instillation of lipopolysaccharide and ultrafine carbon black particles. Experimental and toxicologic pathology : official journal of the Gesellschaft fur Toxikologische Pathologie 61:51-58.

Italiani P, Boraschi D. 2014. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Frontiers in immunology 5:514.

Itaru I, Hiroshi K, Satoshi S, Tomoko S, Sachiko A, Kunihiko I, Sumiko M, Hideaki K, Hidetada S, Takashi K. 2002. Hypoxia Diminishes Toll-Like Receptor 4

Expression Through Reactive Oxygen Species Generated by Mitochondria in Endothelial Cells. J Immunol 169:2069-2075.

Iwasaki A, Medzhitov R. 2015. Control of adaptive immunity by the innate immune system. Nat Immunol 16:343-353.

Janeway CA, Medzhitov R. 2002. Innate immune recognition. Annual Review of Immunology 20:197-216.

Johnson ER, Matthay MA. 2010. Acute lung injury: Epidemiology, pathogenesis, and treatment Journal of Aerosol Medicine and Pulmonary Drug Delivery 23:243–252.

Kawai T, Akira S. 2007. Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med 13:460-469.

Kay RR, Langridge P, Traynor D, Hoeller O. 2008. Changing directions in the study of chemotaxis. Nature reviews Molecular cell biology 9:455-463.

Kennedy AD, DeLeo FR. 2008. Neutrophil apoptosis and the resolution of infection. Immunologic Research 43:25-61.

Khan A, Heit B, Andonegui G, Colarusso P, Kubes P. 2005. Lipopolysaccharide: A p38 MAPK-Dependent Disrupter of Neutrophil Chemotaxis. Microcirculation 12:421-432.

Kim D, Kim YJ, Koh HS, Jang TY, Park HE, Kim JY. 2010. Reactive Oxygen Species Enhance TLR10 Expression in the Human Monocytic Cell Line THP-1. International Journal of Molecular Sciences 11:3769-3782.

Kim SK, Park HJ, Hong IK, Chung JH, Eun YG. 2013. A missense polymorphism (rs11466653, Met326Thr) of toll-like receptor 10 (TLR10) is associated with

tumor size of papillary thyroid carcinoma in the Korean population. Endocrine 43:161-169.

Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, van Rooijen N, van der Poll T. 2003. Alveolar Macrophages Have a Protective Antiinflammatory Role during Murine Pneumococcal Pneumonia. American Journal of Respiratory and Critical Care Medicine 167:171-179.

Kolaczkowska E, Kubes P. 2013. Neutrophil recruitment and function in health and inflammation. Nature reviews Immunology 13:159-175.

Kong L, Ge BX. 2008. MyD88-independent activation of a novel actin-Cdc42/Rac pathway is required for Toll-like receptor-stimulated phagocytosis. Cell Res 18:745-755.

Koppe U, Suttorp N, Opitz B. 2012. Recognition of *Streptococcus pneumoniae* by the innate immune system. Cell Microbiol 14:460-466.

Kumar V, Sharma A. 2010. Neutrophils: Cinderella of innate immune system. Int Immunopharmacol 10:1325-1334.

Lacy P, Stow JL. 2011. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood 118:9-18.

Lambrecht BN, Hammad H. 2015. The immunology of asthma. Nat Immunol 16:45-56.

Lammermann T, Sixt M. 2009. Mechanical modes of 'amoeboid' cell migration. Current opinion in cell biology 21:636-644.

Latz E, Visintin A, Espevik T, Golenbock DT. 2004. Mechanisms of TLR9 activation. Journal of Endotoxin Research 10:406-412.

Latz E, Visintin A, Lien E, Fitzgerald KA, Monks BG, Kurt-Jones EA, Golenbock DT, Espevik T. 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. J Biol Chem 277:47834-47843.

Lazarus R, Raby BA, Lange C, Silverman EK, Kwiatkowski DJ, Vercelli D, Klimecki WJ, Martinez FD, Weiss ST. 2004. Toll-like receptor 10 genetic variation is associated with asthma in two independent samples. Am J Respir Crit Care Med 170:594-600.

Lee JG, Lee SH, Park DW, Lee SH, Yoon HS, Chin BR, Kim JH, Kim JR, Baek SH. 2008. Toll-like receptor 9-stimulated monocyte chemoattractant protein-1 is mediated via JNK-cytosolic phospholipase A2-ROS signaling. Cellular signalling 20:105-111.

Lee KS, Scanga CA, Bachelder EM, Chen Q, Snapper CM. 2007. TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to Streptococcus pneumoniae. Cellular immunology 245:103-110.

Lee VM, Quinn PA, Jennings SC, Ng LL. 2003. Neutrophil activation and production of reactive oxygen species in pre-eclampsia. Journal of hypertension 21:395-402.

Lehrer RI, Ganz G. 1999. Antimicrobial peptides in mammalian and insect host defence. Current Opinion in Immunology 11:23-27.

Leifer CA, Brooks JC, Hoelzer K, Lopez J, Kennedy MN, Mazzoni A, Segal DM. 2006. Cytoplasmic Targeting Motifs Control Localization of Toll-like Receptor 9. Journal of Biological Chemistry 281:35585-35592.

Ley K, Laudanna C, Cybulsky M, Nourshargh S. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 7:678-689.

Li Jeon N, Baskaran H, Dertinger SK, Whitesides GM, Van de Water L, Toner M. 2002. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. Nature biotechnology 20:826-830.

Liu B, Yang Y, Qiu Z, Staron M, Hong F, Li Y, Wu S, Li Y, Hao B, Bona R, Han D, Li Z. 2010. Folding of Toll-like receptors by the HSP90 paralogue gp96 requires a substrate-specific cochaperone. Nature Communications 1:1-10.

Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. 2003. Toll-like Receptor 9-mediated Recognition of Herpes Simplex Virus-2 by Plasmacytoid Dendritic Cells. Journal of Experimental Medicine 198:513-520.

Luo HR, Loison F. 2008. Constitutive neutrophil apoptosis: mechanisms and regulation. American journal of hematology 83:288-295.

MacNee W. 2005. Pathogenesis of chronic obstructive pulmonary disease. Proc Am Thorac Soc 2:258-266; discussion 290-251.

Madhi SA, Klugman KP, Vaccine Trialist G. 2004. A role for Streptococcus pneumoniae in virus-associated pneumonia. Nat Med 10:811-813.

Marcos MA, Esperatti M, Torres A. 2009. Viral pneumonia. Current Opinion in Infectious Diseases 22:143-147.

Marki A, Esko JD, Pries AR, Ley K. 2015. Role of the endothelial surface layer in neutrophil recruitment. J Leukoc Biol.

Marsland BJ, Gollwitzer ES. 2014. Host-microorganism interactions in lung diseases. Nature reviews Immunology 14:827-835.

Martha Triantafilou KM, Douglas T. Golenbock, Triantafilou K. 2002. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci 115:2603-2611.

Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. 2006. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J Leukoc Biol 80:1281-1288.

Mathias W. Hornef, Teresa Frisan, Alain Vandewalle, Staffan Normark, Richter-Dahlfors A. 2002. Toll-like Receptor 4 Resides in the Golgi Apparatus and Colocalizes with Internalized Lipopolysaccharide in Intestinal Epithelial Cells. J Exp Med 195:559-570.

Matthay M, Kronish JW. 1990. Intact epithelial barrier function is critical for the resolution of alveolar edema in humans. Am Rev Respir Dis 142:1250-1257.

Matthay MA, Ware LB, Zimmerman GA. 2012. The acute respiratory distress syndrome. The Journal of clinical investigation 122:2731-2740.

McDonald B, Kubes P. 2011. Cellular and molecular choreography of neutrophil recruitment to sites of sterile inflammation. Journal of molecular medicine 89:1079-1088.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388:394-397.

Meng G, Zhao J, Wang H-M, Ding R-G, Zhang X-C, Huang C-Q, Ruan J-X. 2010. Cell Injuries of the Blood-Air Barrier in Acute Lung Injury Caused by Perfluoroisobutylene Exposure. J Occup Health 52:48–57.

Metersky ML, Masterton RG, Lode H, File TM, Jr., Babinchak T. 2012. Epidemiology, microbiology, and treatment considerations for bacterial pneumonia complicating influenza. Int J Infect Dis 16:e321-331.

Miettinen M, Sareneva T, Julkunen I, Matikainen S. 2001. IFNs activate toll-like receptor gene expression in viral infections. Genes and Immunity 2:49–355.

Miguel R, Wong J, Westoll JF, Brooks HJ, O'Neill LAJ, Gay NJ, Bryant CE, Monie TP. 2007. A Dimer of the Toll-Like Receptor 4 Cytoplasmic Domain Provides a Specific Scaffold for the Recruitment of Signalling Adaptor Proteins. PLoS ONE 2:e788.

Morgan AR, Lam WJ, Han DY, Fraser AG, Ferguson LR. 2012. Genetic variation within TLR10 is associated with Crohn's disease in a New Zealand population. Hum Immunol 73:416-420.

Mouratis MA, Magkrioti C, Oikonomou N, Katsifa A, Prestwich GD, Kaffe E, Aidinis V. 2015. Autotaxin and Endotoxin-Induced Acute Lung Injury. PLoS One 10:e0133619.

Muller WA. 2013. Getting Leukocytes to the Site of Inflammation. Veterinary pathology 50:7-22.

Nelson AL, Roche AM, Gould aM, Chim K, Ratner AJ, Weiser JN. 2006. Capsule enhances Pneumococcal colonization by limiting mucus-mediated clearance. Infect Immun 75:83-90.

Neubauer O, Sabapathy S, Lazarus R, Jowett JB, Desbrow B, Peake JM, Cameron-Smith D, Haseler LJ, Wagner KH, Bulmer AC. 2013. Transcriptome analysis of neutrophils after endurance exercise reveals novel signaling mechanisms in the immune response to physiological stress. Journal of applied physiology 114:1677-1688.

Nicod LP. 2005. Lung defences: An overview Eur Respir Rev 14:45–50.

Nyman T, Stenmark P, Flodin S, Johansson I, Hammarström M, Nordlund P. 2008. The crystal structure of the human toll-like receptor 10 cytoplasmic domain reveals a putative signaling dimer. J Biol Chem 283:11861-11865.

O'Riordan M, Yi CH, Gonzales R, Lee KD, Portnoy DA. 2002. Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. Proceedings of the National Academy of Sciences of the United States of America 99:13861-13866.

O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 374:893-902.

Oh H, Siano B, Diamond S. 2008. Neutrophil isolation protocol. Journal of visualized experiments : JoVE.

Okun E, Lathia JD, Mattson MP. 2009. Adhesion- and migration-related side effects of phosphothioated CpG oligodeoxynucleotides. Cell adhesion & migration 3:272-274.

Oosting M, Cheng SC, Bolscher JM, Vestering-Stenger R, Plantinga TS, Verschueren IC, Arts P, Garritsen A, van Eenennaam H, Sturm P, Kullberg BJ, Hoischen A,

Adema GJ, van der Meer JW, Netea MG, Joosten LA. 2014. Human TLR10 is an anti-inflammatory pattern-recognition receptor. Proceedings of the National Academy of Sciences of the United States of America 111:E4478-4484.

Otero C, Paz RD, Galassi N, Bezrodnik L, Finiasz MR, Fink S. 2013. Immune response to Streptococcus pneumoniae in asthma patients: comparison between stable situation and exacerbation. Clin Exp Immunol 173:92-101.

Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proceedings of the National Academy of Sciences of the United States of America 97:13766-13771.

Palsson-McDermott EM, Doyle SL, McGettrick AF, Hardy M, Husebye H, Banahan K, Gong M, Golenbock D, Espevik T, O'Neill LA. 2009. TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. Nat Immunol 10:579-586.

Parkos CA, Colgan SP, Delp C, Arnaout MA, Madara JL. 1992. Neutrophil migration across a cultured epithelial monolayer elicits a biphasic resistance response representing sequential effects on transcellular and paracellular pathways. J Cell Biol 117:757-764.

Pasparakis M, Vandenabeele P. 2015. Necroptosis and its role in inflammation. Nature 517:311-320.

PHAC. 2011. *Streptococcus pneumoniae* pathogen safety data sheet - Infectious substances. In: Directorate PR, editor. Infectious Substances Canada: Public Health Agency of Canada.

Pollanen R, Sillat T, Pajarinen J, Levon J, Kaivosoja E, Konttinen YT. 2009. Microbial antigens mediate HLA-B27 diseases via TLRs. J Autoimmun 32:172-177.

Pomorska-Mol M, Markowska-Daniel I, Kwit K, Stepniewska K, Pejsak Z. 2013. C-reactive protein, haptoglobin, serum amyloid A and pig major acute phase protein response in pigs simultaneously infected with H1N1 swine influenza virus and Pasteurella multocida. BMC veterinary research 9:14-17.

Price L, McAuley D, PSMarino, Finney S, Griffiths M, Wort S. 2012. Pathophysiology of pulmonary hypertension in acute lung injury. Am J Physiol Lung Cell Mol Physiol 302:L803-L815.

Rebl A, Goldammer T, Seyfert HM. 2010. Toll-like receptor signaling in bony fish. Vet Immunol Immunopathol 134:139-150.

Regan T, Nally K, Carmody R, Houston A, Shanahan F, Macsharry J, Brint E. 2013. Identification of TLR10 as a key mediator of the inflammatory response to Listeria monocytogenes in intestinal epithelial cells and macrophages. J Immunol 191:6084-6092.

Rittirsch D, Flierl MA, Day DE, Nadeau BA, McGuire SR, Hoesel LM, Ipaktchi K, Zetoune FS, Sarma JV, Leng L, Huber-Lang MS, Neff TA, Bucala R, Ward PA. 2008. Acute lung injury induced by lipopolysaccharide is independent of complement activation. J Immunol 180:7664-7672.

Rosenthal LA, Avila PC, Heymann PW, Martin RJ, Miller EK, Papadopoulos NG, Peebles RS, Jr., Gern JE, Infections, Asthma Committee E, Occupational Respiratory Diseases Interest Section AAoAA, Immunology. 2010. Viral respiratory tract infections and asthma: the course ahead. The Journal of allergy and clinical immunology 125:1212-1217.

Sabroe I, Dower S, Whyte M. 2005. The role of toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis 41:S421-S426.

Sabroe I, Parker LC, Dower SK, Whyte MK. 2008. The role of TLR activation in inflammation. J Pathol 214:126-135.

Sanz MJ, Kubes P. 2012. Neutrophil-active chemokines in in vivo imaging of neutrophil trafficking. Eur J Immunol 42:278-283.

Sathiamoorthy S, Hodgins DC, Shewen PE, Highlander SK, Lo RY. 2011. A snap-shot of Mannheimia hemolytica A1 gene expression during infection in the bovine host. FEMS microbiology letters 325:148-154.

Scheel-Toellner D, K W, LK A, PR W, Craddock R, Salmon M. 2004. Clustering of death receptors in lipid rafts inititates neutrophil spontaneous apoptosis Biochem Soc Trans 32:679-681.

Schneberger D, Aharonson-Raz K, Singh B. 2010. Monocyte and macrophage heterogeneity and Toll-like receptors in the lung. Cell and Tissue Research 343:97-106.

Schneberger D, Aharonson-Raz K, Singh B. 2012. Pulmonary Intravascular Macrophages and Lung Health: What Are We Missing? American Journal of Physiology - Lung Cellular and Molecular Physiology.

Schneberger D, Caldwell S, Suri SS, Singh B. 2009. Expression of toll-like receptor 9 in horse lungs. Anatomical record 292:1068-1077.

Schneberger D, Lewis D, Caldwell S, Singh B. 2011. Expression of toll-like receptor 9 in lungs of pigs, dogs and cattle. Int J Exp Pathol 92:1-7.

Scull CM, Hays WD, Fischer TH. 2010. Macrophage pro-inflammatory cytokine secretion is enhanced following interaction with autologous platelets. J Inflamm (Lond) 7:53.

Sedlmayr P, Leintner V, Pilz S, Wintersteiger R, Stewart CC, Dohr G. 2001. Species-Specific Blocking of Fc Receptors in Indirect Immunofluorescence Assays. Lab Hematol 7:81-84.

Sedwick C. 2012. Pruning Brain Vasculature for Efficiency. PLoS Biol 10:e1001375.

Selvarajoo K, Govindaraj RG, Manavalan B, Lee G, Choi S. 2010. Molecular Modeling-Based Evaluation of hTLR10 and Identification of Potential Ligands in Toll-Like Receptor Signaling. PLoS ONE 5:e12713.

Serhan CN, Chiang N. 2004. Novel endogenous small molecules as the checkpoint controllers in inflammation and resolution: entree for resoleomics. Rheumatic diseases clinics of North America 30:69-95.

Shi CS, Kehrl JH. 2010. TRAF6 and A20 regulate lysine 63-linked ubiquitination of Beclin-1 to control TLR4-induced autophagy. Sci Signal 3:ra42.

Simon F, Fernández R. 2009. Early lipopolysaccharide-induced reactive oxygen species production evokes necrotic cell death in human umbilical vein endothelial cells. Journal of hypertension 27:1202-1216.

Singh B, Pearce, J. W., Gamage, L. N., Janardhan, K., & Caldwell, S. 2004. Depletion of pulmonary intravascular macrophages inhibits acute lung inflammation. American Journal of PhysiologyLung Cellular and Molecular Physiology 286:L363-372.

Singh Suri S, Janardhan KS, Parbhakar O, Caldwell S, Appleyard G, Singh B. 2006. Expression of toll-like receptor 4 and 2 in horse lungs. Vet Res 37:541-551.

Smith P. 1994. Ultrastructure of the lung in chronic hypoxia. Thorax 49 Suppl:S27-32.

Snyder LS, Hertz MI, Peterson MS, Harmon KR, Marinelli WA, Henke CA, Greenheck JR, Chen B, Bitterman PB. 1991. Acute lung injury. Pathogenesis of intraalveolar fibrosis. The Journal of clinical investigation 88:663-673.

Standish AJ, Weiser JN. 2009. Human neutrophils kill Streptococcus pneumoniae via serine proteases. J Immunol 183:2602-2609.

Stow JL, Low PC, Offenhauser C, Sangermani D. 2009. Cytokine secretion in macrophages and other cells: pathways and mediators. Immunobiology 214:601-612.

Sundd P, Pospieszalska MK, Cheung LS, Konstantopoulos K, Ley K. 2011. Biomechanics of leukocyte rolling. Biorheology 48:1-35.

Sundd P, Pospieszalska MK, Ley K. 2013. Neutrophil rolling at high shear: flattening, catch bond behavior, tethers and slings. Mol Immunol 55:59-69.

Sutherland ER, Martin RJ. 2007. Asthma and atypical bacterial infection. Chest 132:1962-1966.

Szabo G, Dolganiuc A, Dai Q, Pruett SB. 2007. TLR4, Ethanol, and Lipid Rafts: A New Mechanism of Ethanol Action with Implications for other Receptor-Mediated Effects. The Journal of Immunology 178:1243-1249.

Takeda K, Akira S. 2004. TLR signaling pathways. Seminars in Immunology 16:3-9.

Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. Annu Rev Immunol 21:335-376.

Takeuchi O, Akira S. 2002. Genetic approaches to the study of Toll-like receptor function. Microbes and infection / Institut Pasteur 4:887-895.

Thanawongnuwech R. 1998. The role of pulmonary intravascular macrophages (PIMs) in porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. In: Veterinary Pathology. Iowa: Iowa State University. p 116.

Tizaoui K, Kaabachi W, Hamzaoui K, Hamzaoui A. 2015. Association of Single Nucleotide Polymorphisms in Toll-like Receptor Genes With Asthma Risk: A Systematic Review and Meta-analysis. Allergy, asthma & immunology research 7:130-140.

Togbe D, Schnyder-Candrian S, Schnyder B, Doz E, Noulin N, Janot L, Secher T, Gasse P, Lima C, Coelho FR, Vasseur V, Erard F, Ryffel B, Couillin I, Moser R. 2007. Toll-like receptor and tumour necrosis factor dependent endotoxin-induced acute lung injury. Int J Exp Pathol 88:387-391.

Torraca V, Masud S, Spaink HP, Meijer AH. 2014. Macrophage-pathogen interactions in infectious diseases: new therapeutic insights from the zebrafish host model. Dis Model Mech 7:785-797.

Varin A, Gordon S. 2009. Alternative activation of macrophages: immune function and cellular biology. Immunobiology 214:630-641.

Veltkamp M, van Moorsel CH, Rijkers GT, Ruven HJ, Grutters JC. 2012. Genetic variation in the Toll-like receptor gene cluster (TLR10-TLR1-TLR6) influences disease course in sarcoidosis. Tissue Antigens 79:25-32.

Wang Y, Chen CL, Iijima M. 2011. Signaling mechanisms for chemotaxis. Development, growth & differentiation 53:495-502.

Wassef A, Janardhan K, Pearce JW, Singh B. 2004. Toll-like receptor 4 in normal and inflamed lungs and other organs of pig, dog and cattle. Histol Histopathol 19:1201-1208.

Wegiel B, Larsen R, Gallo D, Chin BY, Harris C, Mannam P, Kaczmarek E, Lee PJ, Zuckerbraun BS, Flavell R, Soares MP, Otterbein LE. 2014. Macrophages sense and kill bacteria through carbon monoxide-dependent inflammasome activation. The Journal of clinical investigation 124:4926-4940.

Wilson R, Cohen JM, Jose RJ, de Vogel C, Baxendale H, Brown JS. 2014. Protection against Streptococcus pneumoniae lung infection after nasopharyngeal colonization requires both humoral and cellular immune responses. Mucosal immunology.

Winkler. 1988. Pulmonary intravascular macrophages in domestic animal species: review of structural and functional properties. Am J Anat 181:217-234.

Wojcik AJ, Skaflen MD, Srinivasan S, Hedrick CC. 2008. A Critical Role for ABCG1 in Macrophage Inflammation and Lung Homeostasis. The Journal of Immunology 180:4273-4282.

Xiang M, Fan J, Fan J. 2010. Association of Toll-like receptor signaling and reactive oxygen species: a potential therapeutic target for posttrauma acute lung injury. Mediators of inflammation 2010.

Yamada H, Arai T, Endo N, Yamashita K, Fukuda K, Sasada M, Uchiyama T. 2006. LPS-induced ROS generation and changes in glutathione level and their relation

to the maturation of human monocyte-derived dendritic cells. Life sciences 78:926-933.

Yi L, Chandrasekaran P, Venkatesan S. 2012a. TLR signaling paralyzes monocyte chemotaxis through synergized effects of p38 MAPK and global Rap-1 activation. PLoS One 7:e30404.

Yi L, Chandrasekaran P, Venkatesan S. 2012b. TLR signaling paralyzes monocyte chemotaxis through synergized effects of p38 MAPK and global Rap-1 activation. PLoS ONE 7:e30404.

Yu L, Wang L, Chen S. 2010. Endogenous Toll-like receptor ligands and their biological significance. Journal of Cell and Molecular Medicine:2592-2603.

Zablockaite D, Gendviliene V, Martisiene I, Jurevicius I. 2007. Effect of oxidative phosphorylation uncoupler FCCP and F1F0-ATPase inhibitor oligomycin on the electromechanical activity of human myocardium. Advances in Medical Sciences 52:89-93.

Zachariae C. 1993. Chemotactic cytokines and inflammation. Biological properties of the lymphocyte and monocyte chemotactic factors ELCF, MCAF and IL-8. Acta Derm Venereol Suppl (Stockh) 181:1-37.

Zhu X, Owen JS, Wilson MD, Li H, Griffiths GL, Thomas MJ, Hiltbold EM, Fessler MB, Parks JS. 2010. Macrophage ABCA1 reduces MyD88-dependent Toll-like receptor trafficking to lipid rafts by reduction of lipid raft cholesterol. J Lipid Res 51:3196-3206.